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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/656,450	09/05/2003	F. Charles Brunicardi	60710-00002USC1	8472

7590 03/03/2006

Tamsen Valoir, Ph.D.
Jenkens & Gilchrist
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1100 Louisiana, Suite 1800
Houston, TX 77002-5214

EXAMINER	
SGAGIAS, MAGDALENE K	
ART UNIT	**PAPER NUMBER**
1632	

DATE MAILED: 03/03/2006

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary	Application No.	Applicant(s)	
	10/656,450	BRUNICARDI, F. CHARLES	
	Examiner	Art Unit	
	Magdalene K. Sgagias	1632	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133) Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☐ Responsive to communication(s) filed on *14 February 2005*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *119-139* is/are pending in the application.

4a) Of the above claim(s) *113-118* is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *119-139* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☒ The drawing(s) filed on *05 September 2003* is/are: a)☒ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08) Paper No(s)/Mail Date *5/13/05*.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☐ Other: _____.

DETAILED ACTION

1. Claims 119-139 are pending.

Claims 113-118 are not entered. Applicants are required to provide a corrected list of all the pending claims in their new application. Appropriate correction is required.

Claims 119-139 are under consideration.

Claim Objections

2. Claims 119, 120, 121, 124, 125, 126, 127, 132 and 136 are objected to because of the following informalities:

Claim 125 and 126 are duplicates of claims 120 and 121. Appropriate correction is required.

Claims 119, 124, 127, 132, and 136 are objected because the phrase "operatively coupled to" is not a recognized art term for a construct composition

Specification

3. The disclosure is objected to because of the following informalities:

In the specification page 1 under prior related applications, Applicant fail to incorporate in line 2 the issued date of the patent number 6716824. Appropriate correction is required.

Double Patenting

4. The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory obviousness-type double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the

examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the conflicting application or patent either is shown to be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement.

Effective January 1, 1994, a registered attorney or agent of record may sign a terminal disclaimer. A terminal disclaimer signed by the assignee must fully comply with 37 CFR 3.73(b).

Claims 119-121, 123-129, 131-134, 136-139 are rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-3 of U.S. Patent No. U.S. 6,716,824. Although the conflicting claims are not identical, they are not patentably distinct from each other because both sets of claims embrace treatment of pancreatic tumor cells in a subject comprising administering to a subject a nucleic acid vector with insulin promoter SEQ ID NO: 1 operatively coupled to a cytotoxic gene wherein the cytotoxic gene in expressed in a pancreatic tumor cell and a prodrug. The breadth of the scope of the claims recited in the instant application is very broad and includes any route of administration of the claimed vector and a prodrug and obviously encompasses the route of direct administration as embraced by the claims 1-3 of U.S. Patent No. U.S. 6,716,824.

Claim Rejections - 35 USC § 112

5. The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claims 119, 124, 127, 132 and 136 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which

was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.

When the claims are analyzed in light of the specification, instant invention encompasses any cytotoxic gene wherein the cytotoxic gene is expressed in a pancreatic tumor cell in a subject. The specification describes that the present invention is directed to an RIP-tk (rat insulin promoter thymidine kinase) construct that targets pancreatic cells (specification p 7). In analyzing whether the written description requirement is met for the genus claims, it is first determined whether a representative number of species have been described by their complete structure. In the instant case, the specification does not provide any disclosure as to what would have been the complete structure of a representative number of cytotoxic genes either within one species or among different species necessary to target pancreatic tumor cells in a subject. While the specification on page 7 lists thymidine kinase as an example, the specification does not provide any disclosure of the structure of any other species of the claimed genus.

Next, then it is determined whether a representative number of species have been sufficiently described by other relevant identifying characteristics, for example other than motif structure, specific features and functional characteristics that would distinguish different members of the claimed genus. In the instant case, the specification fails to describe any identifying characteristics of a cytotoxic gene which will distinguish different species. The specification while listing thymidine kinase as an example of the cytotoxic genes does not provide guidance whether other species of the genus will have any characteristics similar to or different from thymidine kinase.

In conclusion, this limited information is not deemed sufficient to reasonably convey to one skilled in the art that the Applicant is in possession of the cytotoxic gene that will kill a pancreatic tumor cell at the time of the application was filed. Thus it is concluded that the written description requirement is not satisfied for the claimed genus.

Claim Rejections - 35 USC § 112

6. The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claims 119-139 are rejected under 35 U.S.C. 112, first paragraph, because the specification, while being enabling for a method of treating pancreatic adenocarcinoma in a subject comprising directly administering to a subject a nucleic acid comprising a vector with an insulin promoter having SEQ ID NO: 1 operably linked to a cytotoxic gene, wherein the cytotoxic gene is thereby expressed in a pancreatic adenocarcinoma cell wherein administering a prodrug to said subject, wherein the prodrug is converted to a cytotoxic compound by the action of the protein encoded by said cytotoxic gene and thereby killing the pancreatic adenocarcinoma cell wherein the cytotoxic gene is thymidine kinase gene wherein the prodrug is acyclovir, ganciclovir, FIAU or 6-methoxypurine arabinoside, does not reasonably provide enablement for treating a pancreatic tumor in a subject by the way of the claimed method in the instant application. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention commensurate in scope with these claims.

Claims 119-123 are directed to a method of killing a pancreatic tumor cell in a subject by

administering a nucleic acid vector with an insulin promoter SEQ ID NO: 1 operatively coupled to a cytotoxic gene wherein the cytotoxic gene is expressed in a tumor cell that does not express insulin in combination with a prodrug.

Claims 124-126 are directed to a method of treating pancreatic tumor cells in a subject by administering a nucleic acid vector with an insulin promoter SEQ ID NO: 1 operatively coupled to a cytotoxic gene wherein the cytotoxic gene is expressed in a PDX-1 positive pancreatic tumor cell in combination with a prodrug.

Claims 127-131 are directed to a method of killing a pancreatic tumor cell in a subject by administering a nucleic acid vector with an insulin promoter SEQ ID NO: 1 operatively coupled to a cytotoxic gene wherein the cytotoxic gene is expressed in a pancreatic tumor cell in combination with a prodrug.

Claims 132-135 are directed to a method of killing a tumor cell in a subject by administering an adenoviral vector with an insulin promoter SEQ ID NO: 1 operatively coupled to a cytotoxic gene wherein the cytotoxic gene is expressed in a tumor cell expressing PDX-1 in combination with a prodrug.

Claims 136-139 are directed to a method of killing a PDX-1 expressing tumor cell in a subject by administering a nucleic acid vector with an insulin promoter comprising multiple copies of SEQ ID NO: 2 operatively coupled to multiple copies of SEQ ID NO: 3 or 4 wherein insulin promoter operatively coupled to a cytotoxic gene wherein the cytotoxic gene is expressed in PDX-1 expressing tumor cell in combination with a prodrug.

In determining whether a specification is enabling, one considers whether the claimed invention provides sufficient guidance to make and use the claimed invention, if not, whether an artisan would have required undue experimentation to make and use the claimed invention and whether working examples have been provided. When determining whether a specification

meets the enablement requirements, some of the factors that need to be analyzed are; the breadth of the claims, the nature of the invention, the state of the art, the level of one of ordinary skill, the level of predictability in the art, the amount of direction provided by the inventor, the existence of working examples, and whether the quantity of any necessary experimentation to make or use the invention based on the disclosure is "undue" (In re Wands, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed Cir. 1988)). Furthermore, USPTO does not have laboratory facilities to test if an invention will function as claimed when working examples are not disclosed in the specification, therefore, enablement issues are raised and discussed based on the state of knowledge pertinent to an art at the time of the invention, therefore, skepticism raised in the enablement rejections are those raised in the art by artisans of expertise.

These factors are analyzed, in turn, and demonstrate that one of ordinary skill in the art will need to perform "undue experimentation" to make and/or use the invention and therefore, applicant's claims are not enabled.

The specification describes that the present invention is directed to selective targeting of pancreatic cells with cytotoxic genes using promoter driven specific cytotoxic genetic constructs and transcription factors, and to methods for using these constructs and transcription factors to treat cancer and other diseases (specification p 7). The specification more specifically further describes that the present invention is directed to an RIP-tk (rat insulin promoter-thymidine kinase) construct that selectively targets insulin secreting cells, such as beta cells and certain human pancreatic ductal carcinoma cells (PDX-1 positive), to cause death (specification p 7).

While the specification provides teachings pertaining to the ability of RIP-tk suicide gene to target PDX-1 positive human pancreatic ductal carcinoma cells (PANC-1 and CAPAN-1 cells) in vitro and in vivo, and further the cell specific cytotoxicity of human pancreatic ductal carcinoma cells can be achieved using a RIP-tk construct and ganciclovir GVC in vitro and as

well as in in vivo transduced pancreatic tumors using an immunodefficient animal mouse model (specification p 12 and examples 2-4, and figure 8), the specification fails to provide any relevant teachings or specific guidance and/or working examples with regard to: (a) killing of a pancreatic tumor cell that does not express insulin in a subject by administering a construct with an insulin promoter having SEQ ID NO:1 operatively linked to a cytotoxic gene and a prodrug; (b) treating a PDX-1 positive pancreatic tumor cell in a subject by administering a construct with an insulin promoter having SEQ ID NO:1 operatively linked to a cytotoxic gene and a prodrug (c) killing a pancreatic tumor cell in a subject by administering a construct with an insulin promoter having SEQ ID NO:1 operatively linked to a cytotoxic gene and a prodrug; (d) killing a PDX-1 positive tumor cell in a subject by administering an adenoviral vector with an insulin promoter having SEQ ID NO:1 operatively linked to a cytotoxic gene and a prodrug; and (e) killing a PDX-1 positive tumor cell in a subject by administering to a subject a vector with an insulin promoter having SEQ ID NO:2 operatively linked to multiple copies of SEQ ID NO: 3 or 4, said insulin promoter operatively linked to a cytotoxic gene and a prodrug. Thus, as enablement requires the specification to teach how to make and use the claimed invention, the specification fails to enable the claimed methods for killing a pancreatic tumor cell. It would have required undue experimentation to make and use the claimed invention without a reasonable expectation of success.

The claims are directed to methods of killing or treating pancreatic tumor cells in a subject by producing a cytotoxic protein in specific pancreatic tumor cells and clearly fall into the realm of gene therapy. The specification has contemplated killing or treating pancreatic tumor cells using the suicide gene thymidine kinase coupled to an insulin promoter such as the rat insulin promoter followed by treating or administering to the individual an effective amount of GVC, acyclovir, FIAU or 6-methoxypurine arabinoside in an amount sufficient to kill or treat

pancreatic tumors (specification p 17-25). The specification also contemplated that the cytotoxic effect may be enhanced by upregulating transcription of RIP-tk, such as for example, addition of factors that upregulate transcription of RIP-tk as for example SEQ ID NO: 3 or 4 (specification p 19). Since the instant specification has failed to provide specific guidance or working examples correlating to killing or treating pancreatic tumor cells in a subject one of skill in the art could not rely on the state of the gene therapy art to treat any pancreatic tumor cell in a subject by way of the claimed methods. This is because the art of gene therapy is an unpredictable art with respect cell targeting, levels of expression of a therapeutic protein necessary to provide therapy, and mode of administration of the therapeutic gene. These issues are discussed by experts in the field of gene therapy while reviewing the state of the art of gene therapy. Verma et al, (Nature, 389: 239-242, 1998) stated that in gene therapy practice considerable problems have been emerged such as the problem of the inability to deliver genes efficiently and to obtain sustained gene expression (p 239). Anderson (Nature, 392: 25-30, 1998) states that there is still no conclusive evidence that a gene-therapy protocol has been successful in the treatment of a human disease (p 25, 1st column) and concludes, "Several major deficiencies still exist including poor delivery system, both viral and no-viral, and poor gene expression after genes are delivered" (p 30). Applicant's claims do recite adenoviral mediated gene delivery of the cytotoxic gene into a tumor cell expressing PDX-1. The specification however, has not provided any specific guidance or teachings with regard to killing a tumor cell in a subject via adenoviral mediated cytotoxic gene expression encompassed by the claims. The specification does not provide guidance and/or working examples for killing a tumor cell in a subject with a tumor cell expressing PDX-1 by administering adenovirus carrying SEQ ID NO: 1 where the transgene is expressed at levels sufficient and a prodrug to kill target tumor cell by systemic administration. At the time of the instant application, Romano, (Stem

Cells, 17: 191-202, 1999) while reviewing the state of the art of adenoviral vectors noted that while adenoviral vectors can infect nondividing cells there is a need for improvement of adenoviral vector design to deal with the problem of immunogenicity (p 197). While progress has been made in recent years for gene transfer in vivo, vector targeting to desired tissues in vivo continues to be unpredictable and inefficient as supported by numerous teachings available in the art. For example, Romano et al, (Stem Cells, 18: 19-39, 2000) review the latest development in gene transfer technology and noted that despite the latest significant achievements reported in vector design, it is not possible to predict to what extent gene therapeutic interventions will be effective in patients, and in what time frame (abstract). Romano, (Stem Cells, 17: 191-202, 1999) indicate also that gene delivery systems need to be optimized in order to achieve effective therapeutic interventions (abstract). Numerous factors complicate the gene delivery art which would not have been shown to be overcome by routine experimentation. These include, the fate of the DNA vector itself (volume of distribution, rate of clearance into the tissues, etc.), the in vivo consequences of altered gene expression and the protein function, the fraction of vector taken up by the target population, the trafficking of the genetic material within cellular organelles, the rate of degradation of the DNA, the level of mRNA produced, the stability of the mRNA produced, the amount and stability of the protein produced, and the protein's compartmentalization within the cell, or its secretory fate, once produced. These factors differ dramatically based on the vector used and the protein produced. While progress has been made in recent years for the in vivo gene transfer, vector targeting in vivo to desired organs continuous to be unpredictable and inefficient. This is supported by numerous teachings in the art. Deonarain (1998, Exp Opin Ther Patents, 8: 53-69, 1998) indicate that one of the biggest problems hampering successful gene therapy is the "ability to target a gene to a significant population of cells and express it at adequate levels for a long

enough period of time" (p 53, 1st paragraph). Deonarain reviews new techniques under experimentation in the art, which shows promise but states that such techniques are even less efficient than viral gene delivery (p 65, 1st paragraph). Verma reviews vectors known in the art for use in gene therapy and discusses problems associated with each type of vector. The teachings of Verma indicate a resolution to vector targeting has not been achieved in the art (see entire article). Verma also teaches appropriate regulatory elements may improve expression, but it is unpredictable what tissues such regulatory elements target (p 240). In the instant case the specification does not teach as to how to nucleic acid vector having SEQ ID NO: 1, 2, 3 or 4 operatively coupled to cytotoxic gene will be directed to a pancreatic tumor cell in a subject and whether sufficient amount of the cytotoxic gene product could be produced to kill a pancreatic tumor cell in a subject. Furthermore, the specification fails to provide any guidance and/or working examples as to what doses of the claimed nucleic acid vectors will be administered to target a nucleic acid vector to pancreatic tumor cells other than the site of administration. The specification fails to teach one of skill in the art how to overcome the unpredictability for vector targeting such that efficient gene transfer is achieved by either systemic administration or direct administration at the site of the pancreatic tumor cell as claimed in the instant application. It should be noted that although as stated above some publication dates of these cited references is prior to the filing date of the instant application, the issues regarding the unpredictability of gene therapy remain the same and have not be resolved by the guidance provided by the instant specification.

With regard to cytotoxic gene and a prodrug mediated gene therapy for killing or treating a pancreatic tumor cells in a subject as contemplated by the instant specification, the state of the art of a cytotoxic gene and a prodrug mediated gene therapy in pancreatic cancer suggests that while some progress has been made to date there are issues that remain, which make the

treatment of pancreatic cancer by cytotoxic gene and a prodrug mediated gene therapy unpredictable. Nasu et al, (Mol Urol, 4(2): 67-71, 2000) noted that viral-mediated transfer of the herpes simplex virus thymidine kinase (HSV-tk) gene has been demonstrated by several investigators to confer sensitivity to nucleoside analogs such as ganciclovir (GCV) in a variety of tumor cells including pancreas, however, it is still in the early stage of its development, with a number of problems to be overcome such as systemic delivery, specific introduction, and specific expression of the target gene are the major issues to be managed in order to establish a relevant treatment (abstract). MacKenzie (Lancet Oncology, 5: 541-49,2004) wile reviewing the state of the art of gene therapy in pancreatic adenocarcinoma noted that suicide-gene therapy has produced variable results in animal studies on pancreatic cancer and while some studies showed that suicide-gene treatment decreased survival of tumor cells in vitro and in vivo however, other studies have not confirmed the efficacy of suicide genes in pancreatic cell lines (p 542, 2nd column under suicide gene therapy). MacKenzie also noted that although suicide gene approach has not been assessed in patients with pancreatic cancer, results from other tumor sites have not been encouraging (p 542, 2nd column under suicide gene therapy). Fogar et al, (EJSO, 29: 721-730, 2003) noted that suicide gene therapy with HSV-tk did not confer GCV sensitivity to pancreatic cancer in vivo and different pancreatic cancer cell lines cause different growth effects and metastasis patterns after inoculation into SCID mice (abstract). Fogar et al, (Cell Mol Biol, 51(1): 61-76, 2005) even three years later while reviewing killer genes in pancreatic cancer therapy and among them the use of suicide genes (HSV-tk and CD for oancreatic cancer gene therapy in vitro and in vivo noted that the lack of a 100% effect for any studied strategy considered alone, indicates the need for combined therapies to achieve a satisfactory treatment of pancreatic tumor (abstract).

In light of the above, it appears that the state of the art is suggesting that cytotoxic gene

and prodrug gene therapy in pancreatic tumor cells might be feasible in the future. The instant specification does not provide any relevant teachings, specific guidance, or working examples for overcoming the limitations of cytotoxic gene and prodrug gene therapy in pancreatic tumor cells raised by the state of the art. Therefore, the skilled artisan would conclude that the state of art of cytotoxic and prodrug gene therapy in pancreatic tumor cells is undeveloped and unpredictable at best. Given the lack of guidance provided by the instant specification, it would have required undue experimentation to practice the invention as claimed for killing pancreatic tumor cells by cytotoxic gene and prodrug gene therapy without a reasonable expectation of success.

Therefore, in view of the quantity of experimentation necessary to determine the parameters listed above for the cytotoxic gene and prodrug killing of a pancreatic tumor cell in a subject, the lack of direction or guidance provided by the specification for the cytotoxic gene and prodrug killing of a pancreatic tumor cell in a subject, the absence of working examples that correlate to the cytotoxic gene and prodrug killing of a pancreatic tumor cell in a subject, the unpredictable state of the art with respect to the cytoxic gene and prodrug gene therapy, and in particular in pancreatic tumor cells, the undeveloped state of the art pertaining to the cytotoxic gene and prodrug killing of a pancreatic tumor cell in a subject, and the breadth of the claims directed to the cytotoxic gene and prodrug killing of a pancreatic tumor cell that does not express insulin or a pancreatic tumor cell that express PDX-1 or any pancreatic tumor cell in a subject, and also the breadth of the claims directed to cytotoxic gene vectors comprising SEQ ID NO: 1 or SEQ ID NO: 2 or SEQ ID NO: 3, it would have required undue experimentation for one skilled in the art to make and/or use the claimed invention.

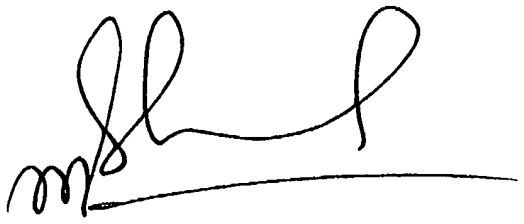
Conclusion

7. No claim is allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Magdalene K. Sgagias whose telephone number is (571) 272-3305. The examiner can normally be reached on Monday through Friday from 9:00 am to 5:00 pm. If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Ram R. Shukla, can be reached on (571) 272-0735. The fax phone number for the organization where this application or proceeding is assigned is (703) 872-9306.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll free).

Magdalene K. Sgagias, Ph.D.
Patent Examiner
Art Unit 1632



RAM R. SHUKLA, PH.D.
SUPERVISORY PATENT EXAMINER

PTO/SB/08a/b (08-03)
Approved for use through 07/31/2006. OMB 0651-0031
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet	1	of	4

Complete If Known	
Application Number	10/656450
Filing Date	September 5, 2003
First Named Inventor	Charles F. Brunicardi
Art Unit	1632
Examiner Name	~~Not Yet Assigned~~ Syagiard
Attorney Docket Number	60710-00002USC1

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
MKS	AA**	US-5,997,859-A1	12-07-1999	Barber et al.	
	AB**	US-6,066,624-A1	05-23-2000	Woo et al.	
	AC**	US-6,241,982B1-A1	06-05-2001	Barber et al.	
	AD**	US-6,217,860B1-A1	04-17-2001	Woo et al.	
	AE**	US-5,674,703-A1	10-07-1997	Woo et al.	
	AF**	US-5,631,236-A1	05-20-1997	Woo et al.	
	AG**	US-5,811,266-A1	09-22-1998	Newgard	
	AH**	US-5,952,221-A1	09-14-1999	Kurtzman et al.	
	AI**	US-5,723,333-A1	03-03-1998	Levine et al.	
	AJ**	US-5,885,971-A1	03-23-1999	German et al.	
	AK**	US-5,880,261-A1	03-09-1999	Waeber et al.	
	AL**	US-5,863,794-A1	01-26-1999	Strayer	
	AM*	US-5,837,693-A1	11-17-1998	German et al.	
	AN**	US-5,837,283-A1	11-17-1998	McDonald et al.	
	AO**	US-5,792,656-A1	08-11-1998	Newgard	
	AP**	US-5,747,325-A1	05-05-1998	Newgard	
	AQ**	US-5,858,973-A1	01-12-1999	Habener et al.	
	AR**	US-5,728,379-A1	03-17-1998	Martuza et al.	

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3]-Number[4]-Kind Code[5] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear	T[6]
MKS	BA**	WO-WO 95/05835-A1	03-02-1995			

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. **CITE NO.: Those patent(s) or publication(s) which are marked with an double asterisk (**) next to the Cite No. are not supplied because they were previously cited by or submitted to the Office in a prior application relied upon in this application for an earlier filing date under 35 U.S.C. 120. [1] Applicant's unique citation designation number (optional). [2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached.

NON PATENT LITERATURE DOCUMENTS

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MKS	CA**	DIMAIO et al., "Directed enzyme pro-drug gene therapy for pancreatic cancer *in vivo*", SURGERY, Vol. 116, No. 2, 1994, pp. 205-213.	
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First Named Inventor	Charles F. Brunicardi
Art Unit	1632
Examiner Name	~~Not Yet Assigned~~ Sgagias
Attorney Docket Number	60710-00002USC1

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First Named Inventor	Charles F. Brunicardi
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Attorney Docket Number	60710-00002USC1

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First Named Inventor	Charles F. Brunicardi
Art Unit	1632
Examiner Name	~~Not Yet Assigned~~ SGorgis
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Gene therapy — promises, problems and prospects

Inder M. Verma and Nikunj Somia

In principle, gene therapy is simple: putting corrective genetic material into cells alleviates the symptoms of disease. In practice, considerable obstacles have emerged. But, thanks to better delivery systems, there is hope that the technique will succeed.

In 1990, the first clinical trials for gene-therapy approaches to combat disease were carried out. Conceptually, the technique involves identifying appropriate DNA sequences and cell types, then developing suitable ways in which to get enough of the DNA into these cells. With efficient delivery, the therapeutic prospects range from tackling genetic diseases and slowing the progression of tumours, to fighting viral infections and stopping neurodegenerative diseases. But the problems — such as the lack of efficient delivery systems, lack of sustained expression, and host immune reactions — remain formidable challenges.

Although more than 200 clinical trials are currently underway worldwide, with hundreds of patients enrolled, there is still no single outcome that we can point to as a success story. To explore why this is the case, we will use our own experience and other examples to look at the many technical, logistical and, in some cases, conceptual hurdles that need to be overcome before gene therapy becomes routine practice in medicine.

At present, gene therapy is being contemplated only on somatic (essentially, non-reproductive) cells. Although many somatic tissues can receive therapeutic DNA, the choice of cell usually depends on the nature of the disease. Sometimes a clear definition of the target cell is needed. For example, the gene that is defective in cystic fibrosis has been identified, and clinical trials to deliver DNA as an aerosol into the lung have already begun[1]. Although cystic fibrosis is manifest in this organ, it is still not clear that delivery of a correcting gene by this method will reach the right type of cell. On the other hand, to correct blood-clotting disorders such as haemophilia, all that is needed is a therapeutic level of clotting protein in the plasma[2]. This protein may be supplied by muscle or liver cells, fibroblasts, or even blood cells[3–5]. The choice of tissue in which to express the therapeutic protein will also ultimately depend on considerations such as the efficiency of gene delivery, protein modifications, immunological status, accessibility and economics.

We also need to consider how much of the therapeutic protein should be delivered. In haemophilia B, which is caused by a deficiency of a blood-clotting protein called factor IX, giving patients just 5% of the normal circulating levels of this protein can substantially improve their quality of life[2]. Most people have about 5 μg of factor IX per millilitre of plasma, produced by the 10^{13} cells that make up the liver. So we need to deliver a correcting gene to 5×10^{11} cells — that is, 5% of liver cells. Alternatively, fewer liver cells would need to be modified if more factor IX could be produced per cell, without being deleterious. In the brain, however, gene transfer to just a few hundred cells could considerably benefit patients with neurological disease. And finally, we can consider the transfer of genes to a handful of stem (or progenitor) cells, which grow and divide to generate millions of progeny. The range in the number of cells that this technology has to cover is vast.

The Achilles heel of gene therapy is gene delivery, and this is the aspect that we will concentrate on here. Thus far, the problem has been an inability to deliver genes efficiently and to obtain sustained expression. There are two categories of delivery vehicle ('vector'). The first comprises the non-viral vectors, ranging from direct injection of DNA to mixing the DNA with polylysine or cationic lipids that allow the gene to cross the cell membrane. Most of these approaches suffer from poor efficiency of delivery and transient expression of the gene[6]. Although there are reagents that increase the efficiency of delivery, transient expression of the transgene is a conceptual hurdle that needs to be addressed.

Most of the current gene-therapy approaches make use of the second category — viral vectors. Importantly, the viruses used have all been disabled of any pathogenic effects. The use of viruses is a powerful technique, because many of them have evolved a specific machinery to deliver DNA to cells. However, humans have an immune system to fight off the virus, and our attempts to deliver genes in viral vectors have been confronted by these host responses. ▶

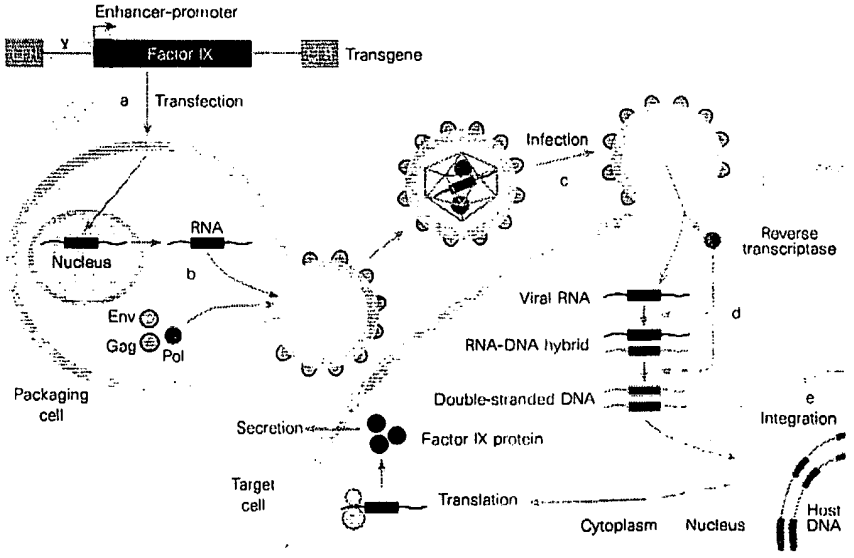

Figure 1 To create the retroviral vectors that are used in gene therapy, the life-cycles of their naturally occurring counterparts are exploited. a, The transgene (in this case, the gene for factor IX) in a vector backbone is put into a packaging cell, which expresses the genes that are required for viral integration (*gag*, *pol* and *env*). b, The transgene is incorporated into the nucleus, where it is transcribed to make vector RNA. This is then packaged into the retroviral vector, which is shed from the packaging cell. c, The vector is delivered to the target cell by infection. The membrane of the viral vector fuses with the target cell, allowing the vector RNA to enter. d, The virally encoded enzyme reverse transcriptase converts the vector RNA into an RNA–DNA hybrid, and then into double-stranded DNA. e, The vector DNA is integrated into the host genome, then the host-cell machinery will transcribe and translate it to make RNA and, in this case, factor IX protein.
LTR, long terminal repeat; ψ, packaging sequence.

Retroviral vectors

Retroviruses are a group of viruses whose RNA genome is converted to DNA in the infected cell. The genome comprises three genes termed *gag*, *pol* and *env*, which are flanked by elements called long terminal repeats (LTRs). These are required for integration into the host genome, and they define the beginning and end of the viral genome. The LTRs also serve as enhancer–promoter sequences — that is, they control expression of the viral genes. The final element of the genome, called the packaging sequence (ψ), allows the viral RNA to be distinguished from other RNAs in the cell (Fig. 1)[7].

By manipulating the viral genome, viral genes can be replaced with transgenes — such as the gene for factor IX (Table 1). Transcription of the transgene may be under the control of viral LTRs or, alternatively, enhancer–promoter elements can be engineered in with the transgene. The chimaeric genome is then introduced into a packaging cell, which produces all of the viral proteins (such as the products of the *gag*, *pol* and *env* genes), but these have been separated from the LTRs and the packaging sequence. So, only the chimaeric viral genomes are assembled to generate a retroviral vector. The culture medium in which these packaging cells have been grown is then applied to the target cells, resulting in transfer of the transgene. Typically, a million target cells on a culture dish can be infected with one millilitre of the viral soup.

A critical limitation of retroviral vectors is their inability to infect non-dividing cells[8], such as those that make up muscle, brain, lung and liver tissue. So, when possible, the cells from the target tissue are removed, grown *in vitro*, and infected with the recombinant retroviral vector. The target cells producing the foreign protein are then transplanted back into the animal. This procedure has been termed '*ex vivo* gene therapy' and our group has used it to infect mouse primary fibroblasts or myoblasts (connective-tissue and muscle precursors, respectively) with retroviral vectors producing the factor IX protein. But within five to seven days of transplanting the infected cells back into mice, expression of factor IX is shut off[3,5,9]. This transcriptional shut-off has even been observed in mice lacking a functional immune system (nude mice), and it cannot be due to cell loss or gene deletion[5] because the transplanted cells can be recovered.

What is the mechanism of this unexpected but intriguing problem? We do not yet know, but the exceptions may provide some clues. To obtain sustained expression in mouse muscle following the transplantation of infected myoblasts, we used the muscle creatine kinase enhancer–promoter to control transcription of the transgene. Unfortunately, this is a weak promoter, and only low levels of protein were produced. So, we generated a chimaeric vector in which the muscle creatine kinase enhancer was linked to a strong promoter from cytomegalovirus. Using this vector, sustained and high levels of factor IX were expressed when the infected myoblasts were transplanted back into mice. Remarkably, these expression levels remained unchanged for more than two years (the life of the animal). So we can override the 'off switch' and achieve higher levels of expression by using an appropriate enhancer–promoter combination. But the search for such combinations is a case of trial and error for a given type of cell.

Another formidable challenge to the *ex vivo* approach is the efficiency of transplantation of the infected cells. Attempts to repeat the long-term myoblast transplantation in haemophiliac dogs led to only short-term expression, because the infected dog myoblasts could not fuse with the muscle fibres. So perhaps successful animal models will prove inadequate when the same protocols are extended to humans. Moreover, these models are based on inbred animals — the outbred human population, with individual variation, will add yet another degree of complexity. The haematopoietic (blood-producing) system may offer an advantage for *ex vivo* gene therapy because resting stem cells can be stimulated to divide *in vitro* using growth factors and the transplantation technology is well established. But there is still a lack of good enhancer–promoter combinations that allow sustained production of high levels of protein in these cells.

Another problem is the possibility of random integration of vector DNA into the host chromosome. This could lead to activation of oncogenes or inactivation of tumour-suppressor genes. Although the theoretical probability of such an event is quite low, it is of some concern (see section on clinical trials).

Lentiviral vectors

Lentiviruses also belong to the retrovirus family, but they can infect both dividing and non-dividing cells[10]. The best-known lentivirus is the human immunodeficiency virus (HIV), which has been disabled and developed as a vector for *in vivo* gene delivery. Like the simple retroviruses, HIV has the three *gag*, *pol* and *env* genes, but it also carries genes for six accessory proteins termed *tat*, *rev*, *vpr*, *vpu*, *nef* and *vif*[11].

Using the retrovirus vectors as a model, lentivirus vectors have been made, with the transgene enclosed between the LTRs and a packaging sequence[12]. Some of the accessory proteins can be eliminated without affecting production of the vector or efficiency of infection. The *env* gene product would restrict HIV-based vectors to infecting only cells that express a protein called $CD4^+$ so, in the vectors, this gene is substituted with *env* sequences from other RNA viruses that have a broader infection spectrum (such as glycoprotein from the vesicular stomatitis virus). These vectors can be produced — albeit on a small scale at the moment — at concentrations of $>10^9$ virus particles per ml (ref. 12).

When lentivirus vectors are injected into rodent brain, liver, muscle, eye or pancreatic-islet cells, they give sustained expression for over six months — the longest time tested so far[13,14]. Unlike the prototypical retroviral vectors, the expression is not subject to 'shut off'. Little is known about the possible immune problems associated with lentiviral vectors, but injection of 10^7 infectious units

Table 1 **Candidate diseases for gene therapy**

Disease	*Defect*	*Incidence*	*Target cells*
Genetic			
Severe combined immunodeficiency (SCID/ADA)	Adenosine deaminase (ADA) in ~25% of SCID patients	Rare	Bone-marrow cells or T lymphocytes
Haemophilia A	Factor VIII deficiency	1:10,000 males	Liver, muscle, fibroblasts or bone-marrow cells
Haemophilia B	Factor IX deficiency	1:30,000 males	Liver, muscle, fibroblasts or bone-marrow cells
Familial hypercholesterolaemia	Deficiency of low-density lipoprotein (LDL) receptor	1:1 million	Liver
Cystic fibrosis	Faulty transport of salt in lung epithelium. Loss of *CFTR* gene	1:3,000 Caucasians	Airways in the lungs
Haemoglobinopathies: thalassaemias/ sickle-cell anaemia	Structural defects in α- or β-globin gene	1:600 in certain ethnic groups	Bone-marrow cells, giving rise to red blood cells
Gaucher's disease	Defect in the enzyme glucocerebrosidase	1:450 in Ashkenazi Jews	Bone-marrow cells, macrophages
α_1-antitrypsin deficiency: inherited emphysema	Lack of α_1-antitrypsin	1:3,500	Lung or liver cells
Acquired			
Cancer	Many causes, including genetic and environmental	1 million/year in USA	Variety of cancer-cell types; liver, brain, pancreas, breast, kidney
Neurological diseases	Parkinson's, Alzheimer's, spinal-cord injury	1 million Parkinson's and 4 million Alzheimer's patients in USA	Direct injection in the brain, neurons, glial cells, Schwann cells
Cardiovascular	Restinosis, arteriosclerosis	13 million in USA	Arteries, vascular endothelial cells
Infectious diseases	AIDS, hepatitis B	Increasing numbers	T cells, liver, macrophages

does not elicit the cellular immune response at the site of injection. Furthermore, there seems to be no potent antibody response. So, at present, lentiviral vectors seem to offer an excellent opportunity for *in vivo* gene delivery with sustained expression.

Adenoviral vectors

The adenoviruses are a family of DNA viruses that can infect both dividing and non-dividing cells, causing benign respiratory-tract infections in humans[11]. Their genomes contain over a dozen genes, and they do not usually integrate into the host DNA. Instead, they are replicated as episomal (extrachromosomal) elements in the nucleus of the host cell. Replication-deficient adenovirus vectors can be generated by replacing the *E1* gene — which is essential for viral replication — with the gene of interest (for example, that for factor IX) and an enhancer–promoter element. The recombinant vectors are then replicated in cells that express the products of the *E1* gene, and they can be generated in very high concentrations ($>10^{11}$–10^{12} adenovirus particles per ml)[15].

Cells infected with recombinant adenovirus can express the therapeutic gene but, because essential genes for viral replication are deleted, the vector should not replicate. These vectors can infect cells *in vivo*, causing them to express very high levels of the transgene. Unfortunately, this expression lasts for only a short time (5–10 days post-infection). In contrast to the retroviral vectors, long-term expression can be achieved if the recombinant adenoviral vectors are introduced into nude mice, or into mice that are given both the adenoviral vector and immunosuppressing agents[16]. This indicates that the immune system is behind the short-term expression that is usually obtained from adenoviral vectors.

The immune reaction is potent, eliciting both the cell-killing 'cellular' response and the antibody-producing 'humoral' response. In the cellular response, virally infected cells are killed by cytotoxic T lymphocytes[16,17]. The humoral response results in the generation of antibodies to adenoviral proteins, and it will prevent any subsequent infection if the animal is given a second injection of the recombinant adenovirus. Unfortunately for gene therapy, most of the human population will probably have antibodies to adenovirus from previous infection with the naturally occurring virus.

It is possible that the target cell contains factors that might trigger the synthesis of adenoviral proteins, leading to an immune response. To try to get around this problem, second-generation adenoviral vectors were developed, in which additional genes that are implicated in viral replication were deleted. These vectors showed longer-term expression, but a decline after 20–40 days was still apparent[18]. This idea has now been taken further with the generation of 'gut-less' vectors — all of the viral genes are deleted, leaving only the elements that define the beginning and the end of the genome, and the viral packaging sequence. The transgenes carried by these viruses were expressed for 84 days[19].

There are considerable immunological problems to be overcome before adenoviral vectors can be used to deliver genes and produce sustained expression. The incoming adenoviral proteins that package DNA can be transported to the cytoplasm where they are processed and presented on the cell surface, tagging the cell as infected for destruction by cytotoxic T cells. So adenoviral vectors are extremely useful if expression of the transgene is required for short periods of time. One promising approach is to deliver large numbers of adenoviral vectors containing genes for enzymes that can activate cell killing, or immunomodulatory genes, to cancer cells. In this case, the cellular immune response against the viral proteins will augment tumour killing. Finally, although immunosuppressive drugs can extend the duration of expression, our goal should be to manipulate the vector and not the patient.

Adeno-associated viral vectors

A relative newcomer to the field, adeno-associated virus (AAV) is a simple, non-pathogenic, single-stranded DNA virus. Its two genes (*cap* and *rep*) are sandwiched between inverted terminal repeats that define the beginning and the end of the virus, and contain the packaging sequence[20]. The *cap* gene encodes viral capsid (coat) proteins, and the *rep* gene product is involved in viral replication and integration. AAV needs additional genes to replicate, and these are provided by a helper virus (usually adenovirus or herpes simplex virus).

The virus can infect a variety of cell types, and — in the presence of the *rep* gene product — the viral DNA can integrate preferentially[20] into human chromosome 19. To produce an AAV vector, the *rep* and *cap* genes are replaced with a transgene. Up to 10^{11}–10^{12} viral particles can be produced per ml, but only one in 100–1,000 particles is infectious. Moreover, preparation of the vector is laborious and, due to the toxic nature of the *rep* gene product and some of the adenoviral helper proteins, we currently have no packaging cells in which all of the proteins can be stably provided. Vector preparations must also be carefully separated from any contaminating adenovirus, and AAV vectors can accommodate only 3.5–4.0 kilobases of foreign DNA — this will exclude larger genes. Finally, we need more information about the immunogenicity of the viral proteins, especially given that 80% of the adult population have circulating antibodies to AAV. These considerations notwithstanding, AAV vectors containing human factor IX complementary DNA have been used to infect liver and muscle cells in immunocompetent mice. The mice produced therapeutic amounts of factor IX protein in their blood for over six months[21,22], confirming the promise of AAV as an *in vivo* gene-therapy vector.

Other vectors

Among the other viruses being considered and developed, is herpes simplex virus, which infects cells of the nervous system[23]. The virus contains more than 80 genes, one of which (*IE3*) can be replaced to create the vector. Around 10^7–10^9 viral particles are produced per ml, but the residual proteins are toxic to the target cell. Additional genes can be deleted, allowing more than one transgene to be included. But if essentially all of the viral proteins are deleted (gut-less vectors), only around 10^7 viral particles are produced per ml. And, again, many people have an immunity to components of herpes simplex virus, having already been infected at some time.

Vaccinia-virus-based vectors have also

What makes an ideal vector?

All of the current methods of gene delivery – whether viral or non-viral – have some limitation. So, the choice of vector will often be dictated by the need. If expression of the gene is required for only a short time (for example, expression of a toxic gene-product in cancer cells), then the adenoviral vectors are ideal. But if sustained expression is needed (such as for most genetic diseases), then an integrating vector without attendant immunological problems is more desirable. An ideal vector may have to borrow properties from both viral and synthetic systems, and it should have:

- High concentration ($>10^8$ viral particles per ml), allowing many cells to be infected;
- Convenience and reproducibility of production;
- Ability to integrate in a site-specific location in the host chromosome, or to be successfully maintained as a stable episome;
- A transcriptional unit that can respond to manipulation of its regulatory elements;
- Ability to target the desired type of cell;
- No components that elicit an immune response.

Although no such vector is currently available, all of these properties exist, individually, in disparate delivery systems.

been explored, largely for generating vaccines[24]. The Sindbis and Semliki Forest virus is being exploited as a possible cytoplasmic vector[25] which does not integrate into the nucleus. Although most of these systems produce the foreign protein only transiently, they add diversity to the available systems of gene transfer (Table 2).

Clinical trials

Over half of the 200 clinical trials that have been launched in the United States involve therapeutic approaches to cancer. Nearly 30 of them involve correction of monogenic diseases (Table 1) such as cystic fibrosis, α_1-antitrypsin deficiency and severe combined immunodeficiency (SCID). Most of the trials are phase I (safety) studies and, by and large, the existing delivery systems have no major toxicity problems. Moreover, lessons can be learned from previous clinical trials[26,27]. For example, seven years ago two patients were enrolled in a trial to correct deficiencies in adenosine deaminase (ADA, which leads to severe immunodeficiency). One of the patients improved, and makes 25% of normal ADA in her T cells, five years after the last infusion of infected T cells (although she is still treated with PEG–ADA; bovine adenosine deaminase mixed with polyethylene glycol). But in the other patient, the infected T cells could not produce enough of the deficient enzyme.

The efficacy of gene therapy cannot be evaluated until patients are completely taken off alternative treatments (in the above example, PEG–ADA). In another trial[28], weaning a patient away from PEG–ADA reduced the ability of the T cells to respond *in vitro* to a challenge by pathogens. Clearly, in these cases the retroviral vectors were not optimal, and the number of infected blood-progenitor cells was extremely low. However, these trials did help to establish the technology for generating clinical-grade recombinant retroviral particles, the procedures for infection and transplantation, and the protocols for monitoring patients and analysing data. The disappointing outcome probably lies in the inefficient gene-delivery system. We need a system in which the vector — containing the ADA gene — is efficiently delivered to progenitor cells, leading to sustained expression of high levels of the ADA protein. But, encouragingly, despite being repeatedly injected with highly concentrated recombinant viruses, the patients have suffered no untoward effects to date.

Future prospects

We now need a renewed emphasis on the basic science behind gene therapy — particularly the three intertwined fields of vectors, immunology and cell biology.

All of the available viral vectors arose from understanding the basic biology of the structure and replication of viruses. Clearly, existing vectors need to be streamlined further (see box on page 241), and vectors that target specific types of cell are being developed. The use of antibody fragments, ligands to cell-specific receptors, or chemical modifications to the vector need to be explored systematically. And advances such as the report — published only last week[29] — of the three-dimensional structure of the Env protein from mouse leukaemia virus (a commonly used vector), will facilitate the design of targeted vectors. A better understanding of gene transcription will allow us to design regulatory elements that permit promoter activity to be modulated, and development of tissue-specific enhancer–promoter elements should be vigorously pursued. Finally, we need to begin merging some of the qualities of viral vectors with non-viral vectors, to generate a safe and efficient gene-delivery system.

With respect to immunology, viruses still have many secrets to be unravelled. Viral systems that have evolved to escape immune surveillance can be incorporated into viral vectors. Some of these are being characterized; for example, the adenoviral E3 protein, the herpes simplex ICP47 protein and the cytomegalovirus US11 protein[30]. Systems from other pathogens may also be borrowed and incorporated into vectors. In some cases, the correcting protein will be sensed as foreign, eliciting an immune response. Animal models should help us to understand this and, where necessary, to develop strategies for tolerance.

Cell biology is involved because, in many cases, the goal of gene therapy is to correct differentiated cells, such as epithelial cells in cystic fibrosis and lymphoid cells in ADA deficiency. However, because these cells are continuously replaced there has to be either continued therapy or an attempt to target the stem cells. We first need to develop further the technologies for identifying and isolating these cells, to understand their regulation, and to target infection to them *in vivo*.

So how far have we come since clinical trials began? The promises are still great, and the problems have been identified (and they are surmountable). But what of the prospects? Our view is that, in the not too distant future, gene therapy will become as routine a practice as heart transplants are today.

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Table 2 **Comparison of properties of various vector systems**

Features	*Retroviral*	*Lentiviral*	*Adenoviral*	*AAV*	*Naked/ lipid-DNA*
Maximum insert size	7–7.5 kb	7–7.5 kb	~30 kb	3.5–4.0 kb	Unlimited size
Concentrations (viral particles per ml)	$>10^9$	$>10^8$	$>10^{11}$	$>10^{12}$	No limitation
Route of gene delivery	*Ex vivo*	*Ex/In vivo*	*Ex/In vivo*	*Ex/In vivo*	*Ex/In vivo*
Integration	Yes	Yes	No	Yes/No	Very poor
Duration of expression *in vivo*	Short	Long	Short	Long	Short
Stability	Good	Not tested	Good	Good	Very good
Ease of preparation (scale up)	Pilot scale up, up to 20–50 l	Not known	Easy to scale up	Difficult to purify, difficult to scale up	Easy to scale up
Immunological problems	Few	Few	Extensive	Not known	None
Pre-existing host immunity	Unlikely	Unlikely, except maybe AIDS patients	Yes	Yes	No
Safety problems	Insertional mutagenesis?	Insertional mutagenesis?	Inflammatory response, toxicity	Inflammatory response, toxicity	None

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Human gene therapy

W. French Anderson

Although gene therapy as a treatment for disease holds great promise, progress in developing effective clinical protocols has been slow. The problem lies in the development of safe and efficient gene-delivery systems. This review will evaluate the problems and the potential solutions in this new field of medicine.

The first approved clinical protocol for somatic gene therapy started trials in September 1990[1]. Since then, in just 7½ years, more than 300 clinical protocols have been approved worldwide and over 3,000 patients have carried genetically engineered cells in their body. The conclusions from these trials are that gene therapy has the potential for treating a broad array of human diseases and that the procedure appears to carry a very low risk of adverse reactions; the efficiency of gene transfer and expression in human patients is, however, still disappointingly low. Except for anecdotal reports of individual patients being helped, there is still no conclusive evidence that a gene-therapy protocol has been successful in the treatment of a human disease. Why not?

In this review I will examine the 'why not?' by evaluating the promise and the problems of gene therapy. There are various categories of somatic cell gene therapy, distinguished by the mode of delivery of the gene to the affected tissue (see Box 1). The challenge is to develop gene therapy as an efficient and safe drug-delivery system. This goal is more difficult to achieve than many investigators had predicted 5 years ago. The human body has spent many thousands of years learning to protect itself from the onslaught of environmental hazards, including the incorporation of foreign DNA into its genome. Viruses, however, have been partially successful in overcoming these barriers and being able to insert their genetic material into human cells. Hence the initial efforts at gene therapy have been directed towards engineering viruses so that they could be used as vectors to carry therapeutic genes into patients. A number of reviews on aspects of gene therapy have been published recently[2–10]; this review will consider the categories of the various virus vectors in turn.

Vectors based on RNA viruses

Retroviruses were initially chosen as the most promising gene-transfer vehicles[11]. Currently, about 60% of all approved clinical protocols utilize retroviral vectors. These RNA viruses can carry out efficient gene transfer into many cell types and can stably integrate into the host cell genome (Fig. 1), thereby providing the possibility of long-term expression. They have minimal risk because retroviruses have evolved into relatively non-pathogenic parasites (although there are exceptions, such as the human immunodeficiency viruses (HIV) and human T-cell lymphotropic viruses (HTLV)). In particular, murine leukaemia virus (MuLV) has traditionally been used as the vector of choice for clinical gene-therapy protocols, and a variety of packaging systems to enclose the vector genome within viral particles have been developed. The vectors themselves have all of the viral genes removed, are fully replication-defective and can accept up to about 8 kilobases (kb) of exogenous DNA.

Box 1 The three categories of somatic cell gene therapy

- The first is *ex vivo*, where cells are removed from the body, incubated with a vector, and the gene-engineered cells are returned to the body. This procedure is usually done with blood cells because they are the easiest to remove and return.
- The second is *in situ*, where the vector is placed directly into the effected tissues. Examples are the infusion of adenoviral vectors into the trachea and bronchi of patients with cystic fibrosis, the injection of a tumour mass with a vector carrying the gene for a cytokine or a toxin, or the injection of a vector carrying a dystrophin gene directly into the muscle of a patient with muscular dystrophy.
- The third is *in vivo*, where a vector could be injected directly into the blood stream. There are no clinical examples of this third category as yet, but if gene therapy is to fulfil its promise as a therapeutic option, *in vivo* injectable vectors must be developed.

The problems that investigators face in developing retroviral vectors that are effective in treating disease are of four main types: obtaining efficient delivery, transducing non-dividing cells, sustaining long-term gene expression, and developing a cost-effective way to manufacture the vector.

Obtaining efficient delivery. Clinical protocols with retroviral vectors primarily use the *ex vivo* approach. Currently, the cells that are transduced by retroviral vectors are those that possess a high level of the natural MuLV (amphotropic) receptor and are actively dividing at the time of exposure to the vector. Most human cells that can be grown *in vitro* can be transduced, although a few cell types cannot. An important target cell is the primitive haematopoietic stem cell (HSC) because gene transfer into these cells would result in gene-engineered cells for the life of the recipient. However, HSCs have a low level of amphotropic receptor and are poorly transducible[12]. The HSC remains, therefore, an important but elusive target.

The broad range of cell types possessing the amphotropic receptor, known to be a phosphate symport, limits the target-specific utility of these vectors in the *in vivo* approach. Using different viral envelope proteins that recognize different receptors (for example, the vesicular stomatitis virus (VSV)-G protein or the gibbon ape leukaemia virus (GALV) envelope protein) can vary the range of cells that can be transduced, but still does not provide much specificity. The difficulty is that, because retroviral vectors cannot be generated at a high titre (amphotropic vectors appear to be limited to 1×10^7 colony-forming units (CFU) per ml and VSV-G pseudotyped vectors to 1×10^9 CFU per ml), it is not possible to get a large number of vector particles to the desired cell type *in vivo*. The viral particles would bind to many cells they encounter and, therefore, would be diluted out before reaching their target (other issues, such as complement-mediated lysis, will be discussed later). The problem can be quantified. The human body contains about 5×10^{13} cells. If a 100 ml sample of retroviral vector were given to a patient, that would be about 1×10^9 active vector particles. Even if every vector particle were 100% efficient at infection, only 1 cell in 50,000 would be transduced. What is needed is a retroviral particle that will preferentially bind to its target cell and can be manufactured at a high titre.

Efforts to target specific cell types have centred on attempts to engineer the natural retroviral envelope protein. The envelope protein has two functions: binding to its receptor (by the surface (SU) moiety) and enabling the entry of the viral nucleoprotein core (carried out primarily by the transmembrane (TM) moiety). The SU protein binds to its receptor on the target cell surface and, as a result, the SU/TM complex undergoes a conformational change that allows fusion of the viral and cellular membranes, followed by entry of the viral core (which carries the virus's genetic information) into the target cell's cytoplasm (Fig. 1).

Two broad approaches to providing target cell specificity have been followed. First, the natural receptor-binding domain of the SU protein has been replaced with a ligand or single-chain antibody that recognizes a specific cell surface receptor[13,14]. A wide range of receptors have

been targeted, but the difficulty is that even though specific binding can be obtained between the engineered vector and the target cell receptor, gene transfer has been unacceptably low in all these experiments. The reason is clear. The retroviral envelope protein is thought to be a trimer with a complex quaternary structure[15]. When the natural receptor-binding domain is replaced by a foreign sequence, the whole structure of the envelope protein is altered. The result is that the natural post-binding conformational change that leads to the fusion of the virus with the cell membrane does not occur. Without fusion, core entry and gene transfer do not take place efficiently.

Engineering the receptor-binding domain of SU while maintaining the ability of the envelope protein to carry out core entry will require a better understanding of the structure–function relationships within the envelope protein complex. This understanding has been enhanced by the recent publication of the three-dimensional structure of the receptor-binding domain of the murine ecotropic (Friend strain) SU protein[16]. It should now be possible to engineer ligands into very specific sites in the SU protein with a higher probability of maintaining the functional properties of the envelope protein for core entry.

Other structure–function studies of the retroviral envelope protein are also contributing to our understanding of how to obtain efficient core entry after binding. The three-dimensional structure of a portion of the Moloney ecotropic retroviral TM protein was published last year[15]. Recently it has been shown that the separate monomers in the predicted trimeric structure of the envelope can cross-talk with each other[17]. In other words, separate monomers, each of which is defective, can complement each other to provide an active trimeric envelope. Using this technique it has been possible to define separate functional domains in the TM protein[18]. As the complete three-dimensional structure and functional domains of the envelope protein become known, constructing retroviral vectors that are able to target specific cells with high efficiency should be possible.

Progress has been made using a second broad approach to targeting that could be called 'tethering'. Although several creative systems have been designed[7], the most successful approach at present appears to be insertion of a ligand that recognizes an extracellular matrix (ECM) component into a part of the SU protein that does not disturb the natural receptor-binding domain. This tethering concentrates the vector in the ECM in the vicinity of the target cells. Receptor binding and core entry can then occur through the natural envelope-receptor mechanism. Two ligands that appear particularly useful for tethering are those specific for fibronectin[19] and for collagen[20]. Fibronectin is present in normal ECM and exposed collagen is present in areas of damage, for example after wound injury as in the cardiovascular system after angioplasty.

Transduction of non-dividing cells. Although the inability of MuLV-based retroviral vectors to transduce non-dividing cells is very useful in some situations, for example when a toxin gene is being inserted into dividing cancer cells and not into the normal non-dividing cells (see below under 'Clinical studies'), there are many situations where one would want to insert a therapeutic gene into normal non-dividing cells. Many potential target cells are not actively dividing *in vivo*; only certain blood cells (not the stem cell) and the cells lining the gastrointestinal tract are continually in division. Lentiviruses (such as HIV-1) are able to infect non-dividing cells, but vectors constructed from these viruses raise concerns over safety because of the possibility that recombination could produce a pathogenic virus. Attempts to transfer into murine retroviral vectors the specific signals from HIV that allow transduction of non-dividing cells have not been successful. Recently it has become possible to use just 22% of the HIV genome (which does not include any of the genes that cause pathology) in a retroviral vector[21,22]. The chances of recombination have been further reduced by the use of a non-HIV envelope protein. This hybrid system holds great promise for providing the option of transducing non-dividing cells *in vivo* in a safe manner. Another RNA viral system being developed is based on the human foamy virus[23]. These vectors are able to transduce a broad range of cell types, are not inactivated by human serum, and may be able to transduce some non-dividing, as well as dividing, cells.

Improving gene expression. Assuming that efficient gene transfer can be developed, the next issue is long-term, stable gene expression at an appropriate level[6]. This is perhaps the greatest shortcoming of present vectors. Although gene expression is being discussed here under retroviral vectors, the topic applies to gene transfer vectors of all types.

Several factors are involved in maintaining the stable expression of genes after their transfer. First, the regulatory sequences that control gene expression often do not remain active. There is a tendency for the cell to recognize foreign promoters (particularly viral promoters such as simian virus 40 (SV40) and cytomegalovirus (CMV)) and inactivate them (by methylation or other mechanisms). The role of lymphokines, cytokines and other growth factors in maintaining gene expression is also poorly understood. Second, even if the gene stays active within the cell, the cell often dies. The immune system is designed to recognize and eliminate foreign gene products and cells that produce a foreign protein. All viral genes are eliminated from retroviral vectors, and so immune recognition of viral proteins (except for those, such as capsid proteins, that are packaged into the viral particle itself) is not an issue (but see the discussion of adenoviral vectors below). Nonetheless, the immune system is still likely to recognize a new or modified protein produced by the therapeutic gene; a newly synthesized normal protein will appear abnormal to an immune system that has never been exposed to it.

Use of a cell's own *cis*-regulatory DNA sequences will probably provide more stable long-term gene expression than can be obtained with viral promoters, but identifying all the components of a gene's regulatory system can be difficult. As an extreme case, the regulatory sequences involved in the proper regulation of the haemoglobin (β-globin) genes are spread over nearly 100 kb. Because a retroviral vector can only accommodate 6–8 kb of sequence, regulatory sequences may need to be truncated to their minimal essential length before being incorporated into such vectors. Even when the natural regulatory elements are used, they may not function correctly without the proper signals and feedback mechanisms that normally operate in the appropriate cellular milieu. For example, the insulin enhancer/promoter still cannot direct regulated expression when delivered to fibroblasts. Again, this emphasizes the need to develop vectors that are capable of gene transfer to specific cell types.

There is steady progress on these fronts, but long-term, stable, appropriate-level gene expression *in vivo* in a range of cell types is still to be accomplished. Once these hurdles are cleared, the next goal will be to achieve gene expression that can be regulated. Many important target genes, such as that for insulin, are not expressed at the same level all the time, but respond to physiological signals within the body. The goal is to use regulatory sequences that respond to the body's own physiological signals (so that inserted therapeutic genes can function the way that normal endogenous genes do) or to drugs that can be used to control the level of gene activity. In some cases, only low levels of essentially unregulated expression may be beneficial (for example, in haemophilia or adenosine deaminase (ADA) deficiency), whereas in other cases tight regulation may be required (for example, for insulin production in diabetes).

Manufacturing the vector. Although consideration of how a pharmaceutical company would be able to manufacture millions of doses of a gene-therapy vector was irrelevant a decade ago, this has now become a real issue. Retroviral vectors are biological agents; they can only be made by living cells. Biological systems are not the easiest systems in which to carry out good manufacturing practice (GMP) and quality assurance/quality control (QA/QC) procedures mandated by the Food and Drug Administration (FDA), as manufacturers of vaccines have learned.

One of the major concerns with retroviral vectors is the possibility that a replication-competent retrovirus (RCR) could arise during the manufacturing process. Because retroviral vectors are produced in

packaging cells that contain a packaging-defective viral genome, and because retroviruses have a high propensity for recombination, this possibility is always present. Furthermore, as every mammalian cell contains endogenous retroviruses, additional viral sequences could be incorporated into the RCR, perhaps producing a pathogenic virus.

Another potential problem results from the ability of retroviral vectors to integrate randomly into host cell DNA. For example, a vector might insert itself into a tumour suppressor gene, thereby increasing the propensity of the cell to become cancerous. The only example of unintentional tumour production in a retroviral gene transfer experiment in large animals was published in 1992; three cases of lymphoma were reported among ten rhesus monkeys whose bone marrow had been destroyed by irradiation and who were then transplanted with haematopoietic stem cells that had been exposed to a large number of RCR as well as the experimental vector[24]. It was shown that the cancers resulted from integration of an RCR (not of the retroviral vector), were clonal events and developed only after a long period (6–7 months) of retroviraemia.

The subject of RCR production and safety as well as of potential tumour production was extensively analysed in a report to the NIH Recombinant DNA Advisory Committee (RAC) and the FDA[25]. The conclusion was that the current QA/QC procedures required by the FDA make it exceedingly unlikely that any patient could receive sufficient RCR to produce either a retroviraemia or a malignancy. However, the manufacturing and testing process to ensure this degree of safety is complex and expensive.

As the goal of present research is the production of a gene therapy vector that can be injected directly into the body (just like penicillin or insulin), additional problems must be considered. For example, mouse packaging cells produce retroviral vectors that are destroyed by human complement. Although this sensitivity makes the vector particles 'safer', it does markedly reduce their half-life *in vivo* and the efficiency of gene transfer. The major component of this sensitivity arises from the presence of unique sugar groups on viral glycoproteins produced in the murine packaging cells that make the viral particles sensitive to human complement. Either the vector particles produced in mouse cells must be engineered to avoid the human complement system, or the vector needs to be made in a non-murine packaging cell line that can provide the viral particles with appropriate sugar groups on their surface. However, as mentioned above, essentially all mammalian cells have their own endogenous retroviruses that could recombine with the vector to produce a new, potentially pathogenic, RCR; many of these endogenous viruses are still unknown. Although any cell line is suspect, the use of primate or human cells as packaging cells raises the greatest safety concerns in this regard. Human packaging cells can, however, be engineered to be very safe. For example, the ProPak cell line[26], which has the viral *gag–pol* genes on a separate DNA construct from the *env* gene (producing a 'split' packaging cell line) as well as other safety features, is certainly safer than the murine packaging cell line PA317, which is used for most of the present retroviral vector clinical trials.

These issues are resolvable, but it will take several years of product development to develop a cost-effective manufacturing system that will produce safe, efficient gene-therapy vectors on a sufficient scale to allow worldwide marketing. Although a non-viral delivery system that avoids many of these problems may be the gene-therapy vector of the future (see discussion below under 'Non-viral vectors'), the many present and future clinical protocols using retroviral vectors require that the manufacturing issues of safety and efficiency be solved.

Vectors based on DNA viruses

Adenoviral vectors. The DNA virus used most widely for *in situ* gene transfer vectors is the adenovirus (specifically serotypes 2 and 5). Adenoviral vectors have several positive attributes: they are large and can therefore potentially hold large DNA inserts (up to 35 kb, see below); they are human viruses and are able to transduce a large number of different human cell types at a very high efficiency (often reaching nearly 100% *in vitro*); they can transduce non-dividing cells; and they can be produced at very high titres in culture. They have been the vector of choice for several laboratories trying to treat the pulmonary complications of cystic fibrosis, as well as for a variety of protocols attempting to treat cancer.

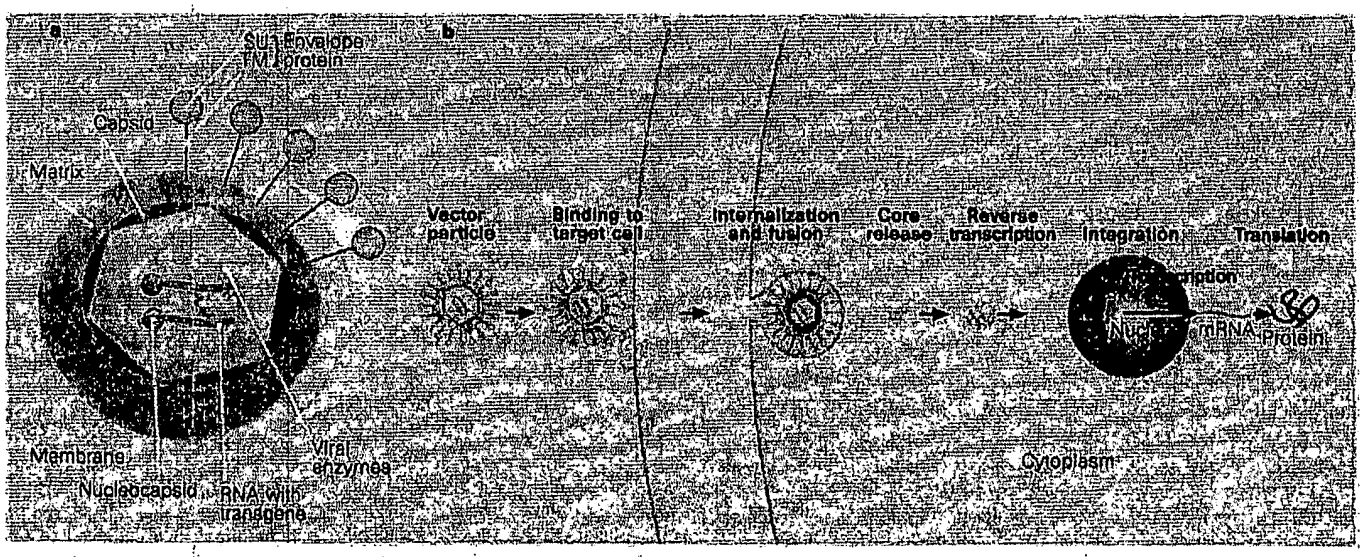

Figure 1 The protocol for retroviral vectors. **a**, Diagram of a retroviral vector. The vector particle is covered by a membrane (derived from the membrane of the cell from which the viral particle budded). Anchored in the membrane is the envelope protein, which is composed of two parts: the SU (surface) protein, which is responsible for binding to the receptor on the target cell, and the TM (transmembrane) protein, which passes through the membrane into the viral matrix and is involved in the fusion step. Beneath the viral membrane is the matrix protein and deeper still is the viral core, which is composed of a surrounding capsid within which are two identical strands of RNA together with the nucleocapsid protein and the viral enzymes (protease, polymerase and integrase). In a retroviral vector the viral genes have been replaced by a transgene. **b**, Diagram of a retroviral vector transducing a target cell. First, the vector particle binds to its receptor on the target cell by means of its SU envelope protein. The particle is then internalized into the cytoplasm of the cell, encased inside an endosome. The envelope protein initiates fusion of the viral membrane with the endosomal membrane, causing the viral core to be released into the cytoplasm. Reverse transcription takes place within the core, which results in the RNA being copied into a double strand of DNA. The double-stranded viral DNA then enters the nucleus, integrates into the chromosomal DNA, and is transcribed. Because the viral genes have been replaced by a transgene, only the protein product of the transgene is made instead of new viral particles.

Adenoviral vectors have certain drawbacks, however. First-generation vectors were deleted for the early region 1 (E1) functions in order to render them replication-defective. In addition, these vectors were deleted in the E3 region in order to create space for the insertion of transgenes. The E3 region, as discussed below, functions to suppress the host immune response during virus infection, but is not required for replication or packaging *in vitro*. Vectors with E1 and E3 deleted elicited strong inflammatory and immune responses[27]. This is thought to be a consequence of 'leaky' expression of adenoviral proteins in the transduced cells because these first-generation vectors retain most of the viral genome. It was hoped that a weaker immune response would result if additional viral genes were deleted. Thus vectors with the deletion of E1 coupled with the deletion of other essential early genes, E2a and/or E4 (refs 28, 29), or vectors with all of the viral genes deleted (so-called 'gutless' vectors[30–32]) have been constructed and tested in animals There have been conflicting reports regarding the immunogenicity, stability of gene expression, and persistence *in vivo* of gutless vectors[33]. In fact, these properties may differ depending on the exact vector design, the tissue type that the vector is introduced into, and the nature of the transgene insert. In particular, the gutted vectors offer the possibility of introducing up to 35 kb of genomic sequences, and it has been suggested that inclusion of nuclear matrix attachment regions might facilitate long-term gene expression and persistence of the vector sequences.

Deleting more and more viral genes may not always be advantageous because some of these genes may have beneficial attributes, for example suppressing an immune response against the vector. Their removal could increase the rate at which the vector is eliminated. As an example, the E3 region encodes a protein of relative molecular mass 19K that protects the virus, and presumably the engineered cells, from host immune surveillance[34]. Various effector mechanisms may be involved in viral vector clearance[35]. In addition, *cis*-acting sequences may exist that help maintain the stability of the adenoviral genome in the cell. As with drug trials, results in animals (even in primates) have not always reflected what happens in patients. Vectors that produce inflammatory responses in primates may not do so in human patients, and the opposite situation is probably also likely. Recently, the first 'true' phase I gene therapy clinical trials have begun: normal volunteers have been tested with intradermal injection (and now by intrabronchial infusion) of adenoviral vectors in order to determine the immunological response to adenoviral vectors in human beings.

By engineering the correct combination of viral genes (incorporating immunosuppressive genes, perhaps from various sources, while deleting immune-stimulating gene products and reducing, if possible, the immunogenicity of viral capsid proteins), it is likely that adenoviral vectors can be generated that have low toxicity, that do not generate an immune response, and that, therefore, can be given repeatedly. The latter point is important because adenoviral vectors do not integrate and they survive in the cell for a limited time (although in non-dividing cells this may be for an extended period). The ability to administer the vector repeatedly will be critical in many treatment protocols, for example in those for haemophilia and cystic fibrosis. Although it would clearly be optimal to engineer vectors that do not elicit an immune response, an interim solution could be to use transient immunosuppression of the patient to allow repeated administration of vectors. Another approach is to blockade costimulatory interactions required for an immune response to an antigen, thereby transiently 'blinding' the immune system during vector administration and making repeat administration possible.

Adeno-associated viral vectors. Another DNA virus used in clinical trials is the adeno-associated virus (AAV). This is a non-pathogenic virus that is widespread in the human population (about 80% of humans have antibodies directed against AAV). Initial interest in this virus arose because it is the only known mammalian virus that shows preferential integration into a specific region in the genome (into the short arm of human chromosome 19). As the virus does not produce disease, its insertion site appears to be a 'safe' region in the genome. It would be useful, therefore, to engineer the sequences that dictate this site-specific insertion into gene-therapy vectors. Unfortunately, the present AAV vectors appear to integrate in a nonspecific manner[36], although it has been suggested that vectors could be designed that retain some specificity[37].

Even though integration site specificity has not been achieved, AAV vectors have been shown to transduce brain, skeletal muscle, liver and possibly CD34$^+$ blood cells efficiently[7,38–40]. There are several drawbacks, however: some cells require a very high multiplicity of infection (the number of viral particles per cell required to achieve transduction); the AAV genome is small, only allowing room for about 4.8 kb of added DNA; and the production of viral particles is still very labour intensive because efficient packaging cells have not yet been developed. However, these vectors hold promise and appear to be safe. Furthermore, AAV may be capable of integrating into non-dividing cells, although again this desirable attribute of the wild-type virus appears to be lost from the vectors, which can enter non-dividing cells but remain in an episomal state until cell division occurs.

Other DNA virus-based vectors

Other DNA viruses are being studied as possible gene-therapy vectors for specific situations. For example, herpes simplex virus (HSV) vectors have a propensity for transducing cells of the nervous system[41,42], as well as several other cell types. A stripped-down version of the HSV, called an amplicon, may have certain advantages, particularly when combined with components from other viral systems[43]. A number of other DNA virus vectors are under study including poxviruses.

Several investigators are examining replication-competent, or attenuated, viral vectors (both DNA and RNA). In addition, hybrid systems have been reported where an adenoviral vector is used to carry a retroviral vector into a cell that is normally inaccessible to retroviral transduction[44].

Non-viral vectors

Although viral systems are potentially very efficient, two factors suggest that non-viral gene delivery systems will be the preferred choice in the future: safety, and ease of manufacturing. A totally synthetic gene-delivery system could be engineered to avoid the danger of producing recombinant virus or other toxic effects engendered by biologically active viral particles. Also, manufacturing a synthetic product should be less complex than using tissue culture cells as bioreactors, and QA/QC procedures should be simplified. The reader is referred to the review on non-viral vectors entitled 'Drug delivery and targeting' by Robert Langer on pages 5–10 of this issue.

Table 1 Disease targets and gene-therapy protocols

(a) Types of gene therapy clinical protocols*

Type	Number	Percentage of total
Therapy	200	(86%)
Marker	30	(13%)
Non-therapeutic†	2	(1%)
Totals	232	(100%)

(b) Disease targets for therapeutic gene therapy clinical protocols

Target	Number	Percentage of total
Cancer	138	(69%)
Genetic diseases	33	(16.5%)
CF	16	
Other‡	17	
AIDS	23	(11.5%)
Others§	6	(3%)
Total	200	(100%)

* Roughly 80% of all protocols use retroviral vectors, 20% use non-viral delivery systems, 10% use adenoviral vectors and the remainder use other viral vectors.
† A 'non-therapeutic' protocol means a non-therapeutic portion of a non-gene-therapy clinical protocol.
‡ These 17 include 12 other monogenic diseases.
§ The five 'other' are: peripheral artery disease, rheumatoid arthritis, arterial restenosis, cubital tunnel syndrome and coronary artery disease (2).

Clinical studies

At present over 300 clinical protocols have been approved. Detailed information is available on the 232 protocols that had been approved in the USA as of 3 February 1998[45] (Table 1).

Only one phase III and several phase II clinical trials are now underway; all the rest of the approved gene therapy clinical protocols are for smaller phase I/II trials. Genetic Therapy Inc./Novartis is carrying out the phase III clinical trial. The target disease is glioblastoma multiforma, a malignant brain tumour[46]. The rationale is to insert a gene capable of directing cell killing into the tumour while protecting the normal brain cells. The retroviral vector used (G1TkSvNa) contains the neomycin-resistance gene as a selective marker and the herpes simplex thymidine kinase (HSTk) gene. The actual material injected into the tumour mass is a mouse producer cell line (PA317) which generates retroviral particles carrying the G1TkSvNa vector. As the only dividing cells in the area of a growing brain tumour are the tumour cells and cells of the vasculature supplying blood to the tumour, and retroviral vectors only transduce dividing cells, the only cells to receive the vector should be the cells of the tumour and its blood vessels. The viral HSTk can add a phosphate group to a non-phosphorylated nucleoside, whereas the endogenous human thymidine kinase cannot. Therefore, when an abnormal nucleoside, such as the drug ganciclovir, is given to the patient, only the cells expressing the HSTk gene will phosphorylate the drug, incorporate it into their DNA synthesis machinery and be killed.

In the current phase III clinical trial, mouse producer cells making vector particles carrying the HSTk gene are inoculated into residual tumour and peritumour areas following tumour resection. After 7 days, the patient is treated with ganciclovir[47]. In theory, the tumour cells that have been transduced with the vector containing the HSTk gene will phosphorylate ganciclovir; the ganciclovir triphosphate then blocks the DNA synthesis machinery and kills the cells.

In fact, at least four distinct mechanisms contribute to tumour cell death in this protocol. First is the direct effect of phosphorylated ganciclovir on the transduced tumour cells; second is the 'bystander' effect in which toxic agents (ganciclovir triphosphate) pass into neighbouring cells through gap junctions and kill them; third, is the local inflammatory effect caused by the injected mouse cells; and fourth is a systemic immune response. The phase III trial includes a total of more than 40 centres in North America and Europe and is scheduled to enrol a total of 250 patients. By the end of December 1997 over 200 patients had been enrolled.

Several phase II trials are underway testing gene-therapy vectors as 'vaccines', either against cancer[48] or against AIDS[49]. Vical has two active phase II trials using a plasmid containing the gene for the HLA-B7/β_2-microglobulin protein formulated with cationic lipids. One trial is for metastatic malignant melanoma and the other for head and neck squamous cell carcinoma. The concept is that an HLA gene (such as B7) that the tumour does not express is injected into the tumour mass and that expression of this foreign antigen should stimulate the immune system to react against the cancer. The data so far suggest that the immune system not only develops a response against the B7 antigen but also to other antigens on the tumour cells, thereby resulting in an immune attack on non-transduced tumour cells[50]. Viagen/Chiron has completed a phase II trial of about 200 patients over 2 years in which a retroviral vector encoding the *env* and *rev* gene segments of the HIV-1(IIIB) strain is injected intramuscularly to induce augmented anti-HIV cytotoxic T-cell responses as a treatment for AIDS. Unfortunately, determination of the efficacy of this treatment was made impossible by the advent of triple drug therapy for HIV infection, but no evidence of toxicity was seen.

Finally, a comment on the original adenosine deaminase (ADA) deficiency gene-therapy trial[51]. ADA deficiency is a rare genetic disorder that produces severe immunodeficiency in children. Starting in 1990, gene-corrected autologous T lymphocytes were given to two girls suffering from this disease. Both girls are doing well and continue to lead essentially normal lives. Patient-1 (A.D.) received a total of 11 infusions, the last being in the summer of 1992. Her total T-cell level and her level of transduced T cells have remained essentially constant for the past 5½ years. She contracted chickenpox in late 1996 and experienced the same clinical course as would have been expected for any normal 10-year-old. Both she and patient 2 (C.C.) continue to receive polyethylene glycol (PEG)-ADA. Although both girls have gene-engineered T lymphocytes in their circulation after more than 7 years, no definitive conclusion can be drawn as to the relative roles of PEG-ADA and gene therapy in their excellent clinical course.

Ethical issues

Somatic cell gene therapy for the treatment of serious disease is now accepted as ethically appropriate. Indeed, it is so well accepted, and the side effects from gene-therapy protocols have been so minimal, that the danger now exists that genetic engineering may be used for non-disease conditions, that is for functional enhancement or 'cosmetic' purposes. The first Gene Therapy Policy Conference organized by the NIH RAC focused on this issue in September 1997. The conclusion was that enhancement engineering is about to take place, and could slip through the regulatory process if RAC and the FDA (and similar organizations in other countries) are not vigilant. As an example, a US biotechnology company has developed the technology for transferring genes (specifically the tyrosinase gene) into hair follicle cells[52]. They are now looking for genes that promote hair growth with the clinical objective of reversing the hair loss that occurs after chemotherapy in cancer patients. The application to the FDA for product licensing would list chemotherapy-induced alopecia as the product indication. The risk–benefit analysis here would be very favourable. However, once a product is licensed for any indication, it can be prescribed by physicians for any 'off-label' use that is felt by the physician to be clinically justified. The result could be millions of balding men receiving gene therapy to treat their hair loss. The conference concluded that the FDA should use a risk–benefit analysis that takes into account the extensive off-label usage for cosmetic reasons that could take place.

Using genetic engineering to treat baldness is not a major issue in itself, of course. But this is just one example of how our society is moving towards a slippery slope where genetic engineering might very well be used for a broad range of enhancement purposes, including larger size from a growth hormone gene, increased muscle mass from a dystrophin gene and so on. If we knew that there would be no long-term negative effects of genetic engineering, then widespread, or even frivolous, use of genetic engineering technology might not be detrimental. But just as with nuclear energy, pesticides and fluorocarbons, we as a society tend to see the benefits but are caught off guard by the bad effects of our powerful new technologies. What society wants to do 100 years from now with regards to genetic engineering is their business, but it is our duty to begin the era of genetic engineering in as responsible a manner as possible. Until we have learned about the long-term effects of somatic cell gene therapy in the treatment of disease, we should not use this technology for any other purpose than where it is medically indicated[53].

In utero somatic gene therapy of the fetus will be undertaken in the foreseeable future. The same care should be exercised here as with somatic cell gene-therapy protocols for adults, children and newborns. So long as only serious disease is targeted and the risk–benefit ratios for both mother and the fetus are acceptable, *in utero* gene therapy should be ethically appropriate[54]. Germline gene therapy should not be attempted at this time for the reasons outlined elsewhere[55].

A situation with the potential for real abuse of the new technologies would be the combination of cloning and genetic engineering. This combination has already been achieved in sheep where single cells have been obtained from fetal fibroblasts, transduced with a gene (human factor IX), and the gene-engineered cells grown into living sheep producing human factor IX[56]. Attempts to use such techniques to produce genetically engineered humans would provoke an even greater ethical storm than the present suggestion by a Chicago scientist to clone humans.

The future

The ultimate goal of gene-therapy research is the development of vectors that can be injected, will target specific cells, will result in safe and efficient gene transfer into a high percentage of those cells, will insert themselves into appropriate regions of the genome (or will persist as stable episomes), will be regulated either by administered agents or by the body's own physiological signals, will be cost-effective to manufacture and will cure disease. As the number of target cells may be in the billions, very high efficiency of gene transfer and the injection of a large number of gene-therapy vectors may be necessary. How soon can we expect significant progress in each of these areas?

The next 5 years should bring the first successes for gene therapy, that means statistically significant data that a gene-therapy protocol results in significant improvement in the clinical condition of patients. Within this time frame the first vectors that can target specific tissues should begin clinical trials and tissue-specific gene expression should have made its way into clinical trials.

In a time frame of 5–15 years from now, I expect that the number of gene-therapy products will begin to increase exponentially, coinciding with the enormous increase in characterized genes as a result of the Human Genome Project. The first injectable vectors will reach clinical trials and efficient tissue-specific gene transfer will be available in a few cases. It will probably take longer to develop site-specific integration, efficiently regulated genes and the correction of genes *in situ* by means of homologous recombination. Beyond this, our imagination is the limit.

For many gene-therapy applications in the future, it is probable that a synthetic hybrid system will be used that incorporates engineered viral components for target-specific binding and core entry, immunosuppressive genes from various viruses and some mechanism that allows site-specific integration, perhaps utilizing AAV sequences or an engineered retroviral integrase protein. In addition, regulatory sequences from the target cell itself will be utilized to allow physiological control of expression of the inserted genes. All these components would be assembled *in vitro* in a liposome-like formulation with additional measures taken to reduce immunogenicity such as concealment by PEG.

Conclusions

Gene therapy is a powerful new technology that still requires several years before it will make a noticeable impact on the treatment of disease. Several major deficiencies still exist including poor delivery systems, both viral and non-viral, and poor gene expression after genes are delivered. The reason for the low efficiency of gene transfer and expression in human patients is that we still lack a basic understanding of how vectors should be constructed, what regulatory sequences are appropriate for which cell types, how *in vivo* immune defences can be overcome, and how to manufacture efficiently the vectors that we do make. It is not surprising that we have not yet had notable clinical successes. Nonetheless, the lessons we are learning in the clinic are invaluable in illuminating the problems that future research must solve.

Despite our present lack of knowledge, gene therapy will almost certainly revolutionize the practice of medicine over the next 25 years. In every field of medicine, the ability to give the patient therapeutic genes offers extraordinary opportunities to treat, cure and ultimately prevent a vast range of diseases that now plague mankind. □

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Concise Review

Latest Developments in Gene Transfer Technology: Achievements, Perspectives, and Controversies over Therapeutic Applications

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ABSTRACT

Over the last decade, more than 300 phase I and phase II gene-based clinical trials have been conducted worldwide for the treatment of cancer and monogenic disorders. Lately, these trials have been extented to the treatment of AIDS and, to a lesser extent, cardiovascular diseases. There are 27 currently active gene therapy protocols for the treatment of HIV-1 infection in the USA. Preclinical studies are currently in progress to evaluate the possibility of increasing the number of gene therapy clinical trials for cardiopathies, and of beginning new gene therapy programs for neurologic illnesses, autoimmuno diseases, allergies, regeneration of tissues, and to implement procedures of allogeneic tissues or cell transplantation. In addition, gene transfer technology has allowed for the development of innovative vaccine design, known as genetic immunization. This technique has already been applied in the AIDS vaccine programs in the USA. These programs aim to confer protective immunity against HIV-1 transmission to individuals who are at risk of infection. Research programs have also been considered to develop therapeutic vaccines for patients with AIDS and generate either preventive or therapeutic vaccines against malaria, tuberculosis, hepatitis A, B and C viruses, influenza virus, La Crosse virus, and Ebola virus. The potential therapeutic applications of gene transfer technology are enormous. However, the effectiveness of gene therapy programs is still questioned. Furthermore, there is growing concern over the matter of safety of gene delivery and controversy has arisen over the proposal to begin in utero gene therapy clinical trials for the treatment of inherited genetic disorders. From this standpoint, despite the latest significant achievements reported in vector design, it is not possible to predict to what extent gene therapeutic interventions will be effective in patients, and in what time frame. *Stem Cells 2000;18:19-39*

INTRODUCTION

The advent of gene transfer technology in therapy marks its tenth anniversary. The first phase I gene-based clinical trial dealt with the treatment of adenosine deaminase deficiency, and is now a milestone of experimental medicine [1]. The initial success of this clinical trial prompted the submission of many other human gene therapy protocols. Over the last decade, more than 300 phase I and phase II gene therapy clinical trials have been conducted worldwide for the treatment of cancer [2-4], and of inherited or acquired genetic disorders [3, 4]. The aim of these clinical trials was mainly to assess the degree of toxicity of the various gene delivery systems and the constructs employed in the study. The possible therapeutic efficacy of the clinical trials was only a secondary issue, which in many cases could not even be determined because of the preliminary nature of the study design. In many cases the cohorts of patients were already terminally ill at the time of the gene therapy intervention, especially where cancer patients were being treated.

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Already in the early 1990s, the first phase of enthusiastic pursuit of gene therapy programs was soon followed by a certain degree of skepticism. The level of vector design development was not adequate enough to meet all the enthusiastic expectations of the investigators who were involved in gene therapy programs at that time [5]. In the following years, research strategies were cautiously planned and an enormous effort was dedicated towards the improvement of vector design [5]. In this matter, significant advances have recently been realized, and they are discussed in this review. The standpoint of the current gene therapy research programs clearly indicates both the presence of a sober optimism among scientists, and a more active role of gene transfer technology in clinical trials [6-9] for the treatment of cancer [2, 3], inherited or acquired monogenic disorders [3], and AIDS [3]. Indeed, gene therapy is one of the fastest growing areas in experimental medicine. As of June 1998, in the USA there were 244 gene therapy clinical trials that were either active or in the evaluation phase by the Recombinant DNA Advisory Committee (RAC) of the National Institutes of Health (NIH) [3]. Most of these trials were either phase I or phase II. In March 1999, the RAC reported that 248 human gene therapy protocols were registrated at the Office of Recombinant DNA Activities. Interestingly, 173 of these trials are for treatment of cancer, 36 for monogenic disorders (mainly cystic fybrosis), 27 for AIDS and 12 for unspecified other disorders (this information is available at http://www.nih.gov/od/orda/). This picture reflects the worldwide trend. This contrasts with the early predictions on the fate of gene therapy, which was thought to be primarily employed to treat inherited or acquired genetic disorders. Such a dramatic change of pattern is certainly motivated by the technical difficulties experienced in establishing long-term transgene expression in humans [10], which is one of the most critical requirements for the successful correction of monogenic disorders [5]. In addition, gene therapy clinical research for the treatment of inherited or acquired genetic diseases is much less likely to receive support from pharmaceutical industries, due to the rarity of these illnesses. Undoubtedly, pharmaceutical companies are interested in developing gene therapy programs for the treatment of more common pathological conditions. This explains, in part, the increase in the number of cancer gene therapy protocols, the beginning of AIDS gene therapy programs, and the sudden development of preclinical studies for gene-based treatments of cardiovascular diseases and neurologic disorders. It has been estimated that about 3,500 patients have been enrolled worldwide in gene-based clinical trials. It is expected that this figure will increase in the next few years. However, the real effectiveness of gene therapy programs is still in question. After a decade of clinical trials, the therapeutic applications of gene transfer technology are still at a rather preliminary stage [5]. Despite the latest improvements reported in the area of vector design, viral and nonviral-based vectors are not sufficiently developed to allow for a large scale application of gene therapy in phase III clinical trials. The purpose of these trials is to assess the therapeutic efficacy of the various treatments in patients. Preclinical studies are currently in progress to test the level of improvement in the various gene delivery systems. The potential applications of gene transfer technology in therapy are enormous. As anticipated, the spectrum range of the pathological conditions that can potentially be treated comprise cancer [2], inherited or acquired monogenic disorders [3, 4], AIDS [3], and other infectious diseases. Most likely, this spectrum will soon be extended to cardiopathies [5, 10, 11], neurologic illnesses [12-14], regeneration of tissues [15-17], and allergies [18]. Indeed, some gene therapy clinical trials have already been carried out for the treatment of cardiovascular diseases [19-21]. In addition, gene transfer technology is emerging as a powerful tool for innovative vaccine design, which has been termed genetic immunization [5, 22-32]. The vectors are either based on viral or nonviral gene delivery systems, and on mechanic administration of naked DNA. The AIDS vaccine programs, which began in the USA in 1996, have widely adopted these gene-based immunization techniques in phase I and phase II clinical trials [22-24, 33], in attempts to confer protective immunity to individuals at risk of HIV-1 infection. Studies are currently in progress to generate more viral vectors to be used in the AIDS vaccine programs [34], and develop therapeutic vaccines for patients with AIDS [35]. Several research programs are addressing the possibility of developing either preventive or therapeutic DNA-based vaccines against malaria [29, 30, 32, 36, 37], tuberculosis [26, 32], hepatitis A, B and C viruses [38-45], influenza virus [27, 28], Ebola virus [31], and La Crosse virus [46]. Interestingly, the same principle of genetic immunization may be used to treat allergies [18] and autoimmune diseases [47, 48], or to prevent the rejection of transplanted allograft tissues [49, 50].

Another line of intervention that has recently been proposed is in utero gene therapy. These clinical trials envisage the treatment and/or prevention of certain inherited genetic disorders, which may have catastrophic outcomes in children. This proposal has raised concern and conflict within the scientific comunity [51-53]. For the moment, the RAC has not authorized in utero gene therapy clinical trials, as there are not sufficient data from animal model systems to ensure the safety of this procedure in humans.

There are several motivations for interest in establishing gene therapy and genetic immunization programs. The pursuit of these programs implies an enormous effort by

scientists and clinicians. Despite the latest progress reported in the area of vector design, research strategies still have to tackle critically important issues, such as further improvement of gene transfer technology, especially for in vivo gene delivery applications, regulation and control of the transgene expression post-cell transduction, and a variety of complex safety matters. These three main issues are to some extent intertwined and pose severe limitations on the applications of gene transfer technology in therapy.

Achievements in Vector Design

The successful realization of gene therapy programs in medicine is highly dependent upon the degree of vector design development. This area of investigation has to deal with a number of pressing and complex issues in order to optimize the performance of gene transfer technology in preclinical studies and clinical research. The aspects that need to be addressed may be summarized as follows:

- The transduction efficiency of both viral- and nonviral-based vectors must be improved. Also, the production and purification procedures for vectors must be optimized.
- In the matter of gene delivery safety, the first rule is that vectors must not be pathogenic or toxic to the patients. For this reason, viral vectors have been engineered to be noncompetent for replication, and devoid of viral factors that may pose a hazard in humans. However, a great deal of attention is still drawn to the possibility of replication-competent virus formation in patients. Another concern is the issue of insertional mutagenesis of vectors based on retroviruses or on adeno-associated virus (AAV) type 1 or type 2. A rather new aspect that has been considered is the possible recombination between retroviral-based vectors and human endogenous retroviruses (HERVs). In order to improve the performance of gene transfer technology, viral-based vectors must be modified in order to reduce their toxicity and immunogenicity in patients. A number of significant advances have been accomplished in this respect. One study has also raised some concern about the immunogenicity of selectable markers [54], which normally derive from bacteria. Therefore, the transduction of cells of the hematopoietic lineages may lead to selectable markers entering the antigen-presenting cell pathway. This in turn would render the transduced cells susceptible to cytotoxic T lymphocyte (CTL) immune responses [54]. Indeed, this principle is the very basis of genetic immunization.
- It is necessary to enhance the targeting and specificity of vectors to avoid unpredictable side effects due to the ectopic expression of the transgene in normal tissues. This requirement is essential to generate gene delivery systems suitable for in vivo administration. Most of the human gene therapy protocols currently rely on ex vivo gene transfer manipulations, in which certain cells or tissues must be removed from the patient, transduced in vitro, possibly selected for the expression of the transgene, and then reinfused into the patient. The entire procedure is costly and distressful for the patient. Health care systems and pharmaceutical companies would greatly benefit from the possibility of applying gene therapy approaches based on in vivo gene delivery, as the therapeutic interventions are minimally invasive, and may only require either an injection or the administration of pills [18]. Indeed, the in vivo transduction approach would also allow for a broader application of gene transfer technology in therapy. Certain pathological conditions cannot be dealt with using the ex vivo gene therapy approach, as not all cells or tissues can be surgically removed. Neurons or cardiac cells are an example. However, the in vivo gene therapy approach poses many additional safety concerns versus the ex vivo one. Recent studies have shown there is a possibility that exogenous DNA (transgene and/or viral vector sequences) may eventually be transmitted to the germ line through systematic in vivo administration of viral vectors [55, 56]. Sensitive nested polymerase chain reaction (PCR) techniques have allowed for the detection of low levels of exogenous viral vector DNA in the ovaries and testes of mice, which received systematic administration of adenoviral vectors [56]. Ninety-four percent of these animals tested positive for the presence of adenoviral DNA in the gonads. However, after mating the animals there was no evidence of germ line transmission of adenoviral DNA in the offspring [56]. This issue should also be addressed for in vivo retroviral- or AAV-mediated gene transfer. These viral vectors may have higher probabilities of entering the germ line, as they integrate their chimeric viral genome into host chromosomal DNA [5].
- In many cases, the possibility of regulating transgene expression following cell transduction would be a highly desirable feature. This should allow for the activation of a transgene when it is needed, the maintenance of transgene expression within a therapeutic window, and the possibility of silencing a transgene if necessary. There have been a number of attempts to generate inducible systems. Partial successes have been reported in the in vitro system [5, 57-62] and animal models [63-67]. However, whether transgene

regulation can be achieved in patients is still an open question.

- The possibility of combining gene-based interventions with other therapeutics has to be considered.

A broad arsenal of gene transfer systems is currently avalaible [5] and is still in expansion. The characteristics of the main vector systems are described in Table 1. Each gene delivery system has distinct characteristics and preferential applications in therapy [5]. The vectors that have already been applied in clinical trials are based on retroviruses [68-72], adenovirus [73-78], AAV [79-85], vaccinia virus [86, 87], canarypox virus [87], herpes simplex virus (HSV) [88], cationic liposomes [89-92], polylysine-DNA complexes [93, 94], and injection of naked DNA [22, 26, 27, 30]. As anticipated, the pathological conditions with which gene therapy has dealt so far comprise: cancer [2], inherited or acquired monogenic disorders [3, 4], AIDS [3], and cardiovascular diseases [19-21]. In addition, vectors based on vaccinia virus, canarypox virus, injection of naked DNA and other nonviral vectors have been used in the AIDS vaccination programs in the USA [22-24]. Interestingly, viral-based vectors have also been directly administered to patients in order to transduce in vivo cells that are capable of processing the transgene through the antigen-presenting cell pathway. In these cases, the transgene encodes for certain HIV-1 components. The intracellular expression of viral antigens within transduced cells facilitates the cells' antigen-presenting mechanism. In this way various viral epitopes are associated with host HLA class I antigens and expressed on the cell membrane to elicit the host's CTL immune responses [5].

Preclinical tests have been carried out to characterize the gene delivery properties of vectors based on foami virus [95-97], lentiviruses (such as HIV-1 [98-104] and feline immunodeficiency virus (FIV) [105-107]), human cytomegalovirus (CMV) [108], Epstein-Barr virus [109], negative-strand RNA viruses (influenza virus) [110], alphaviruses [111], herpesvirus saimiri [112], hybrid adenoviral/retroviral vector systems [113, 114], and hybrid alphavirus/retroviral vector [115]. Other preclinical studies are also assessing the level of vector design improvement that has been reported for a variety of gene transfer models.

Vector Systems Based on Retroviruses, Lentiviruses and Foami virus

Retroviruses have attracted a great deal of interest from the standpoint of gene transfer applications [5]. Such interest is certainly motivated by the characteristics of the biology of retroviruses, which belong to the genera of the *retroviridae*. This category also comprises lentiviruses and foami viruses. The *retroviridae* have a long history of cross-species infections [116, 117]. They have been responsible for many zoonotic events (transmission of infectious agents from animals to humans) [116] which indicates that they may be suitable for DNA delivery into humans. The retroviral genome is relatively simple [118], so it may easily be rearranged to generate recombinant viral vector particles which are noncompetent for replication [5], and which can sustain only one round of infection. Retroviral vectors are mainly based on the amphotropic Moloney murine leukemia virus (MLV) [118], and have been used in many gene therapy clinical trials for the treatment of cancer [2, 5], inherited or acquired monogenic disorders [5], and AIDS [119-124]. Lentiviral vectors are based on HIV-1 [98-104] or on FIV [105-107]. Neither lentiviral- or foami virus-based vectors have been used in clinical trials yet. However, the HIV-1-based lentiviral vector system is unlikely to be approved for clinical trials for a variety of reasons. First is the issue of the serum conversion of the patients to HIV-1. Secondly, is the production and administration of lentiviral vector stocks require category three facilities. Third, the large quantities of lentiviral vector stocks that have to be produced for the clinical trials pose an additional concern in the matter of biosafety. Fourth, this vector system is already obsolete, due to the development of the FIV-based lentiviral vector system, which has circumvented all the above-mentioned issues. In fact, FIV has been certified for category two manipulations, and is based on a lentivirus which cannot infect humans. Therefore, the serum conversion to FIV does not raise any concern. The characteristics of the *retroviridae* vector systems are summarized in Table 1. All these viral vector systems can be produced at relatively high titers (10^6-10^7 cfu/ml) [5]. A property of retroviruses is that they can only infect dividing cells, as they need the breakdown of the nuclear membrane to be able to deliver the preintegration complex into the cell nucleus [125]. Conversely, lentiviruses [98-107] and, to a lesser extent, foami viruses [95-97, 126] can also infect nondividing cells. The requirement for active cell division can be either an advantage or a drawback for retroviral vectors. The selective transduction of dividing cells makes retroviral vectors suitable for cancer therapy [5]. On the other hand, retroviral vectors cannot be used for a variety of therapeutic applications, such as neurologic diseases and a number of genetic diseases that require the transduction of hepatocytes [127], as neurons and hepatocytes do not divide. In all these respects, FIV-based lentiviral vectors may find useful applications. Indeed, retroviral vectors have been used in a number of preclinical studies for liver-directed gene transfer and in some clinical trials [127]. The procedure used was based on ex vivo or in vivo transduction of hepatocytes, which were induced to proliferate by complex and artificial procedures [127]. Retroviral-mediated ex vivo transduction relies on stimulating cell division by culturing primary hepatocytes in appropriate

Table 1. Description of the main gene delivery systems

Vectors	Characteristics	Disadvantages and possible adverse effects in therapy
Retroviruses	• Relatively high titers (10^6-10^7 cfu/ml) • Broad cell tropism • Stable gene expression due to viral genome integration into cell chromosomes • No toxic effect on infected cells • Total insert capacity in the virion is in the range of 10kb (transgene + transfer vector) • They only infect dividing cells	• Random insertion of viral genome, which may possibly result in mutagenesis • Possibility of replication competent virus formation by homologous recombination • Retroviral vector particles are rapidly degraded by the complement • Possible recombination with human endogenous retroviruses (HERVs)
HIV-1-based lentivirus	• It can infect nondividing cells • It can be pseudotyped with retroviral or VSV G envelopes, therefore it also has broad cell tropism • Stable gene expression due to viral genome integration into cell chromosomes • Relatively high titers (10^6-10^7 cfu/ml) • Total insert capacity in the virion is in the range of 10kb (transgene + transfer vector)	• Serum conversion to HIV-1 • Possible proviral insertional mutagenesis in target cells • Presence of tat and rev regulatory proteins, (the early lentiviral vectors also have some HIV-1 accessory proteins) • Possible recombination with human endogenous retroviruses (HERVs) • Possibility of replication competent virus formation by homologous recombination
FIV-based lentivirus	• It can infect nondividing cells • It can be pseudotyped with retroviral or VSV G envelopes, therefore it also has broad cell tropism • Stable gene expression due to viral genome integration into cell chromosomes • Relatively high titers (10^6-10^7 cfu/ml) • Total insert capacity in the virion is in the range of 10kb (transgene + transfer vector)	• Possible proviral insertional mutagenesis in target cells • Presence of FIV regulatory proteins in the early vectors • Possible recombination with human endogenous retroviruses (HERVs) • Possibility of replication competent virus formation by homologous recombination
Foami virus (human or simian)	• It infects dividing cells; nondividing cells are also infected, but to a lesser extent • Foami virus is resistant to lysis mediated by complement • It can be pseudotyped with retroviral or VSV G envelopes, therefore it also has broad cell tropism • Stable gene expression due to viral genome integration into cell chromosomes • Relatively high titers (10^6-10^7 cfu/ml) • Total insert capacity in the virion is in the range of 14kb (transgene + transfer vector)	• Possible proviral insertional mutagenesis in target cells • Possible recombination with human endogenous retroviruses (HERVs) • Serum conversion to foami virus. A number of zoonotic events have occurred among animal care workers, and, so far, there is no evidence that the foami virus is pathogenic in humans, and that it can be transmitted among humans • Possibility of replication competent virus formation by homologous recombination
Adenoviruses	• Very high titers (10^{13} pfu/ml) • Transiently high levels of gene expression • They can also infect nondividing cells • Large DNA inserts can be accommodated in the vector (7-8kb can be simply added to the adenoviral vector; larger DNA inserts can be added, provided that an equivalent part of the viral genome is properly deleted)	• Host immune responses: inflammatory and toxic reactions in patients and depletion of transduced cells • Host's humoral immune responses may neutralize adenoviral vector particles during, or even before, the gene transfer processes • Not suitable for long-term expression of the transgene due to the lack of integration into host genome • Complicated vector genome
Adeno-associated viruses (AAV)	• Wide range of cells can be infected, including cells which do not divide • High titers (10^{10} cfu/ml) • Ability of the virus to establish latent infection by viral genome integration into cell genome • Viral integration specific for human chromosome 19 (only for wild-type AAV) • Nonpathogenic, nontoxic • Small genome (5kb)	• High titers of pure virus are difficult to obtain • AAV requires a helper adeno- or herpesvirus • This vector system is still not well characterized • Limited capacity for foreign genes (about 4kB) • Lack of specific integration for recombinant AAV vectors, which may possibly result in cell mutagenesis
Herpes simplex virus (HSV)	• Titers are in the range of 10^7 to 10^8 cfu/ml • The maximum size of the transgene can reach 30kb • It does not integrate into the cell genome • It allows for long-term expression of the transgene in neuronal cells • It induces cytopathic effects in cancer cells	• Host immune responses, inflammatory and toxic reactions in patients • Complicated vector genome
Cationic liposomes or DNA-protein complexes	• They are not infectious • Theoretically, there is no limit to the size of DNA • They are suitable for oligonucleotide delivery • Low degree of toxicity	• Targeting is not specific • Low transfection efficiency • Only transient expression • Difficult in vivo applications • Host immune responses, inflammatory reactions in patients if they express chimerical cell receptors on their surface, or in the presence of unmethylated CpG sequences of bacterial plasmid DNA

media [127]. This approach has been employed in preclinical studies for the following genetic diseases: type I tyrosinemia, familial hypercholesterolemia and α_1-antitripsin deficiency [127]. One clinical trial was conducted to treat familial hypercholesterolemia by retroviral-mediated ex vivo gene transfer. The low-density lipoprotein (LDL) receptor gene was introduced into hepatocytes that had been surgically removed from patients, and which were then reinfused into the liver following gene transduction [128, 129]. The procedure was safe but there was no convincing evidence of therapeutic efficacy [127]. Liver biopsies were removed after treatment, and few cells tested positive for the expression of LDL-receptor [127], indicating that the transduction efficiency was not high, or that transduced cells were lost or eliminated after reinfusion into the liver. In vivo retroviral-mediated transduction of hepatocytes is even more complicated, as it requires artificial regeneration of the liver [127]. This may be achieved by a variety of means: partial hepatectomy, chemical injury, administration of growth-stimulating drugs or vascular occlusion [127]. Experiments in animal models have shown efficient retroviral-mediated gene transfer into the liver of rodents [127], but a poor efficacy of intervention in larger animals such as dogs [127]. This is probably due to the different kinetics of liver regeneration between large mammals and rodents. In conclusion, in vivo administration of retroviral vectors into the liver does not seem applicable to humans. Probably, the development of a retroviral vector system based on the hepatitis B virus may facilitate liver-directed gene delivery. In this respect, a hepatitis B-based retroviral vector is under development [130, 131]. Interestingly, one study has shown successful liver-directed hepatitis B viral-mediated gene transfer of green fluorescence protein. In addition, the delivery of type I interferon by hepatitis B-based retroviral vector has suppressed endogenous wild-type virus replication in the duck model of hepatitis B virus infection [131]. However, this viral vector system needs further characterization, and should also be adapted to the rodent animal model before considering its application in clinical trials.

All the viral vectors based on *retroviridae* can be used to transduce a wide range of cell types. This is due to the fact that HIV-1, FIV and foami virus cores can be pseudotyped with the MLV amphotropic envelope or vesicular stomatitis virus G (VSV G) glycoprotein (Table 1) [132, 133]. Pseudotyping with the VSV G glycoprotein also allows for easy purification of the various viral vector particles, as they became more stable and resistant, so they can be isolated from the cell culture supernatants by simple ultracentrifugation [134]. Foami viral vectors have a broad cell tropism, even without being pseudotyped with MLV amphotropic envelopes or with VSV G glycoprotein [95-97, 126]. Interestingly, wild-type foami viruses are resistant to complement-mediated lysis [95] and have a total insert capacity in the virion of approximately 14kb [95]. Conversely, MLV-based retroviral, lentiviral and foami viral vectors pseudotyped either with amphotropic retroviral envelopes or VSV G glycoprotein are susceptible to complement-mediated lysis [135-138] and their total insert capacity in the virion is in the range of 10kb [5]. It has been demonstrated that packaging cell lines expressing galactosyl(alpha1-3)galactosyl (alphaGal) sugars generate enveloped viruses that are more susceptible to complement attachment [136]. The viral systems analyzed in this study were based on VSV, HIV-2 and human foami virus [136]. It has been argued that the humoral immune response to alphaGal may be a mechanism of defense against the transmission of viral agents from animals to humans [136], and that viral vectors for human gene therapy should be produced from alphaGal-negative cells [136]. Another study has reported the production of MLV-based amphotropic retroviral vectors resistant to human complement [139]. This was achieved by expressing hybrid amphotropic envelopes on the viral membrane. These hybrid amphotropic envelopes were generated by fusing in frame the catalytic domain of the human complement regulatory protein decay-accelerating factor with a portion of the envelope [139].

The possibility of concentrating retroviral, lentiviral, and foami viral vector particles may improve the transduction efficiency for both ex vivo and in vivo applications. The protection from complement-mediated lysis is particularly required for the optimization of in vivo gene transfer models. A number of other studies have been conducted to further improve the performance of retroviral vectors in preclinical studies and clinical trials. A simple approach consists of using enhanced green fluorescence protein as reporter gene [140-143]. This allows for the rapid detection and isolation of the fraction of cells that have been transduced ex vivo. In addition, the green fluorescence protein can be readily detected in tissues following infusion of transduced cells into the animals [140, 141]. Other strategies to improve the retroviral transduction efficiency are based on the artificial induction of cell division. This can be achieved in many ways: preincubation of primary cultures of hematopoietic stem cells with various interleukins (IL-2, IL-3, IL-6) and/or other growth factors or colony-stimulating factors [144-146]; combination of retroviral- and lipofectAMINE-mediated gene transfer into stem cells prestimulated with IL-2 (in this study, lipofectAMINE was used to facilitate the delivery of retroviral vectors into the target cells) [147]; colocalization of retroviral particles and hematopoietic stem cells on specific fibronectin fragments (Retronectin) [148]; combination of Retronectin system with prestimulation of hematopoietic stem cells with ILs or other growth factors [149, 150]. Ex vivo retroviral transduction of human hematopoietic stem cells also has several disadvantages. Besides being costly and time-consuming, this

approach may introduce some artifacts into hematopoietic stem cells. For instance, the in vitro culture conditions may impair the ability of transduced hematopoietic stem cells to engraft once they are reinfused into the subject. This situation has already been mentioned for the gene-based clinical trial for the treatment of familial hypercholesterolemia, in which the target cells were hepatocytes [127]. The tissue culture conditions for the ex vivo propagation and transduction of human hematopoietic stem cells are conducted at nonphysiologic cell concentrations, and require the combination of growth factors that may induce cell differentiation and, therefore, pose a limitation to the long-term engraftment of the transduced cells. It has been observed that HIV-1- and FIV-based lentiviral vectors may be more suitable for the transduction of hematopoietic cells than amphotropic retroviral vectors [98, 100, 104, 107]. The ability of lentiviruses to also infect nondividing cells may circumvent the issue of prestimulating hematopoietic stem cells [151]. Moreover, lentiviruses usually yield higher transduction efficiency of primary stem cell cultures than retroviral vectors [152, 153]. However, an important aspect that must be addressed in the matter of lentiviral-mediated gene transfer is to establish whether the transfer vector remains episomal in the nucleus of transduced cells that are in G_0 phase. Transgene expression detected following lentiviral transduction of quiescent cells may indeed derive from extrachromosomal double-stranded DNA transfer vector. If this is the situation, lentiviral transduction of quiescent cells may only allow for transient expression of the transgene.

An important safety issue in the matter of viral-mediated gene transfer is the formation of viral-competent viruses in patients, which may occur by homologous recombination events within the packaging cell lines. Retroviral vector stocks are routinely monitored in clinical trials for the absence of replication-competent retroviruses (RCR) [154]. The techniques are essentially based on sensitive PCR and serological enzyme-linked immunosorbent assay [154]. In addition, retroviral stocks must be tested for the absence of endotoxins and various contaminating agents, such as bacteria and fungi, which may be acquired during the propagation of packaging cell lines or target cells [119, 155]. The purity of the various genetic material used in the trial must also be tested [119, 155]. The RCR formation is a rather unlikely event due to the design of retroviral vector. The current trend is to produce high titer retroviral vector stocks transiently [5] in order to further minimize the possibility of recombination events among the various retroviral components in the packaging cell line. These transient systems are based on three plasmid cotransfections of the highly transfectable 293T cell line [156]. As reviewed elsewhere [5], the proviral genome has been broken down into three parts, and overlapping sequences have been mostly removed. The RCR formation is unlikely due to the fact that it would require simultaneous rearrangement among three different plasmids in a specific configuration in a very limited period of time. The transfection procedure usually takes between 48 to 72 h to produce the retroviral vector stocks [5]. So far, the retroviral vectors used in clinical trials derive from conventional packaging cell lines, which were previously approved for clinical applications by the U.S. Food and Drug Administration [3, 157]. Studies are currently addressing the issue of generating clinical grade retroviral vector stocks by transient transfection systems [158].

Another safety concern is the possible recombination between retroviral vectors and HERVs in patients (Table 1). The human genome contains thousands of HERV sequences [159-161], most of which are defective genes. These HERV sequences derive from ancient retroviral infections [160] in which transmission occurred either in germ line cells or cells in the early embryo [160, 161]. About 1% of the human genome is composed of HERV-related sequences [161], and probably more than 10% of the human genome may have evolved through reverse transcription mechanism [161]. So far, only one HERV has been found that encodes for a complete viral particle, which was named HERV-K [162]. However, HERV-K is not competent for replication [162]. The biological relevance of HERVs deserves further investigation. HERVs have some possible advantageous effects in fundamental biological processes such as: development and/or differentiation, protection from superinfection by exogenous retroviruses, protection of the embryo from retroviral infection (germ line vaccination), cell fusion, tissue-specific gene expression, alternative splicing, and polyadenylation [161]. The potential pathogenicity of HERVs cannot be predicted. They may be involved in the development of malignancies and autoimmune diseases [161]. The envelope of an HERV may either protect the host from exogenous retroviral infection in a receptor interference fashion [163] or dysregulate the local cellular immunity through a superantigen-encoded region, as proposed for type I diabetes [164]. A study has observed that the multiple sclerosis-associated retrovirus detected in the plasma of patients with multiple sclerosis [165, 166] has high homology to an HERV [167], which was named HERV-W. Xenotransplantation techniques and gene therapy approaches based on *retroviridae* vectors may eventually tamper with the biology of HERVs [161]. Retroviral vectors may recombine with HERVs in patients, and generate a variety of possible adverse effects. At this point in time, we cannot predict possible adverse effects of recombination due to the lack of sufficient information about HERVs. What one can expect is the formation of RCR in patients, or the expression of HERV genes that were silent prior to gene therapy or xenotransplantation intervention. If such events should occur, most likely the subject may develop cancer or become susceptible to immune system dysregulation.

The integration of the retroviral genome into chromosomes allows for stable transgene expression. This stability is also due to the low degree of retroviral particle immunogenicity. This is in contrast with what has been observed for adenoviral-mediated gene transfer, where transgene expression is only transient. There are two reasons for the transient nature of transgene expression in adenoviral-mediated gene transfer. First, the adenoviral genome does not integrate into the host chromosomal DNA [5]. Second, the adenoviral particles are immunogenic [5] and express leaky adenoviral genes that render the transduced cells susceptible to CTL immune responses [168-171]. Stable retroviral-mediated transgene expression is desirable for the treatment of diseases that require long-term expression of the transgene, such as genetic disorders and neurologic illnesses [5]. However, the duration of transgene expression is still not optimal. This is because the retroviral long terminal repeats (LTRs) are susceptible to methylation in CpG-rich islands, which may silence the gene transcription [172, 173]. The incidence of this phenomenon depends on the type of transduced cells and the site of retroviral genome integration [174]. It has been shown that Sp1 binding sites may, to some extent, prevent the methylation of the promoter [175]. Retroviral vectors based on murine embryonic stem cell virus (MESV) [176, 177] and on murine stem cell virus (MSCV) [178] have been engineered to optimize the duration of transgene expression in undifferentiated murine embryonic and hematopoietic cells [176-178]. To this end, the LTRs of the MESV- and of the MSCV-based vectors have been modified. In the MESV vectors, the 5′-LTR contains an extra Sp1 binding site, which has been introduced by a point mutation [176, 177]. This has optimized, to some extent, the duration of transgene expression in embryonic and hematopoietic cells. However, silencing of transcription has been observed following the differentiation of embryonic stem cells [179]. The MSCV-based vectors, in addition to the point mutation that creates an Sp1 binding site, contain another point mutation that destroys the binding site of the embryonal LTR-binding protein (ELP) [178]. ELP is a transcriptional suppressor of the activity of the MLV 5′-LTR in undifferentiated murine embryonal carcinoma cells [180]. These modifications have further improved the performance of retroviral vectors in terms of duration of transgene expression. However, better evaluation of the exact extent of this improvement in in vivo systems is needed.

The random insertion of the retroviral transfer vector has several drawbacks: it may damage the cell genome, cause the inactivation of tumor suppressor genes, or induce the expression of cellular oncogenes. Probably, this is not sufficient to generate a neoplasia, as cancer is a multistep process which requires a combination of genetic alterations and the expression of cellular and/or exogenous oncogenic factors [181]. However, if the transduced cells should be genetically impaired by the random insertion of the viral vector's genome, this would at least predispose the cells to undergo to neoplastic transformation. To date, human gene therapy protocols have been applied only to a limited number of patients, and most of them did not have a long life expectancy. An important question is what happens if retroviral-mediated gene transfer is applied to larger scale clinical trials and subjects who have a life expectancy in the range of some decades? The current development of preventive cancer prognosis cannot answer this question, so it is not possible to properly assess the ratio of benefit to risk for all the patients. A recent study has addressed the issue of cell transformation induced by retroviral-mediated gene transfer in an in vitro system [182]. Mouse fibroblasts BALB/c-3T3 cells were transduced with a retroviral vector, and the transformation frequency was compared to that of the untransduced cells [182]. The parental cell line undergoes spontaneous transformation that is in the range of 1.1×10^{-5} [183]. In this study, the transformation rate of retrovirally transduced BALB/c-3T3 cells was in the same range [182]. The number of integrated proviral copies per cell genome varied from one to six, depending on transduction efficiency [182]. So, improved transduction efficiency is correlated with better transgene expression, which in turn is due to the higher number of integrated retroviral transfer vector's copies per cell genome. But this is also proportional to the higher risk of mutagenic events. Previous studies on retroviral-induced mutagenesis in mammalian cells have found a ratio of "mutations versus insertional events" which ranged from 10^{-9} to 10^{-3} [184-189]. Such variability indicates that the ratio of mutations per insertional events depends on the cell type and assay system. This ratio should be established for human primary lymphocytes, which normally are not retrovirally transduced as efficiently as mouse fibroblasts [182, 190, 191] or other cultured cell lines [191]. However, the lower transduction efficiency, per se, does not guarantee a lower ratio of "mutations versus insertional events" in human primary lymphocytes. All these findings suggest that in the design of clinical protocols using retroviral-mediated gene transfer, the average number of integrated viral genomes should be carefully evaluated. Such a procedure is feasible for ex vivo retroviral-mediated gene transfer, but not for the in vivo administration system.

Overall, the in vivo administration of retroviral vectors poses a number of additional safety concerns and technical limitations if compared to the ex vivo gene transfer model. To pursue the goal of safe and efficient in vivo retroviral transduction, it is necessary to generate tissue- or cell-specific retroviral vectors, which can integrate their genome in

safe cell chromosomal sites. The latter issue has never been tackled, whereas the engineering of ecotropic-based retroviral vectors with altered cell tropism has attracted much attention [5], but all the attempts had little success. The chimeric retroviral particles that have been produced have a low transduction capacity [5], or even fail the gene transfer process [192]. To date, the ex vivo retroviral-mediated gene transfer model is more realistic than the in vivo one, although it is not optimal for gene therapy applications. Also, from the standpoint of safety concern, the ex vivo procedure can be more easily monitored.

Adenoviral Vectors

There has been a remarkable increase in gene therapy clinical trials based on adenoviral-mediated gene transfer over the last three years [193]. For instance, of 170 gene therapy clinical trials registered with the NIH RAC in 1996, only 15% relied on adenoviral vectors [193]. In the following two years, 91 new human gene therapy protocols were submitted to the NIH RAC, and 32% of them were based on adenoviral-mediated gene transfer [193]. This finding reflects the improvement in adenoviral vector design, which has allowed for a wider application of adenoviral-mediated gene transfer in preclinical studies and therapy. As previously reviewed [5], the first recombinant adenoviral vectors were engineered in 1985, and were based on serotype 2 or 5 [73-75], due to the fact that these two adenoviral serotypes are not associated with severe illnesses and do not cause tumors in animals, in contrast to the other serotypes. The first applications of adenoviral vectors in gene therapy clinical trials were conducted in the early 1990s for the treatment of patients with cystic fibrosis [194]. Now, adenoviral vectors are widely employed in human cancer gene therapy [2] and some other somatic gene therapy clinical trials [195]. Adenoviral vectors have a number of positive characteristics (Table 1): they can also transduce nondividing cells; they can be produced at very high titers of 10^{10} pfu/ml, and easily concentrated to 10^{12} pfu/ml; it is possible to achieve high levels of transgene expression, but only transiently; and they have a large insertional capacity for foreign genes, which is in the range of 7-8 kb (about 6% of the wild-type adenoviral genome). If the genome of the adenoviral vector is properly deleted, it is possible to accomodate into the virion DNA fragments even bigger than 7-8 kb, depending on the size of the deletion [5]. In addition, adenoviral vectors based on serotype 5 may be excellent for liver-directed gene therapy approaches, as the adenovirus serotype 5 is preferentially localized in the liver post-intravenous injection of rodents [196, 197]. All these properties make adenoviral vectors a very attractive gene delivery system, which could potentially be employed in a variety of pathological conditions, such as neurologic disorders, cardiopathies, inherited or acquired monogenic diseases, and cancer. Unfortunately, the current design of adenoviral-mediated gene transfer is affected by some drawbacks, which severely limit the applications of adenoviral vectors in preclinical studies and therapy (Table 1). Firstly, adenoviral vector particles are highly immunogenic in the host [5]. This is a significant obstacle to improving adenoviral vector design. Besides eliciting inflammatory and toxic reactions in the host, immunogenicity is also responsible for the depletion of adenovirally transduced cells [198-203], and may also reduce the efficacy of adenoviral-mediated gene transfer readministration to the patients. In fact, adenoviral particles are also susceptible to humoral immune responses, which may neutralize most of the vector load before the gene transfer is carried out [204]. The issue of humoral immune responses is even more complicated in patients, as, in most cases, they already have an acquired immunity to adenoviruses prior to the gene therapy intervention. Immunogenicity derives from leaky expression of adenoviral early genes (E1, E2, E3 and E4) following adenoviral cell transduction [5]. The deletion of E1 gene is essential for generating replication-defective adenoviral vectors [205]. E1 functions are supplied in trans by a cell line that is stably transformed with the adenoviral E1 gene [206]. Secondly, the transgene expression can only be transient, because adenoviruses do not integrate their genome into the cellular chromosomial DNA [5, 207]. If such an event should occur, it would just be fortuitous [204]. Therefore, both immunogenicity and lack of adenoviral genome integration into the host's chromosomal DNA contrive to suppress long-term transgene expression of adenoviral-mediated gene transfer. Taken together, these properties limit the application of adenoviral-mediated gene transfer for the treatment of pathological conditions that require a long-term transgene(s) expression, such as inherited or acquired monogenic disorders, neurologic illnesses and cardiovascular diseases. From the standpoint of gene-based cancer therapy, adenoviral vectors are useful, provided that inflammatory and other toxic reactions in patients are carefully monitored. One goal of cancer therapy is the selective destruction of neoplastic tissues and cells. This may be accomplished by a variety of means [2], and on a transient basis, followed by systematic administration of adenoviral vectors carrying the appropriate transgene. Another advantage of adenoviral vectors in cancer gene therapy is that they can transduce neoplastic cells regardless of their mitotic status. In most of the cancer gene therapy clinical trials, adenoviral vectors have been administrated in vivo [2], and have been used to deliver drug-sensitivity genes, such as the herpes virus timidine kinase [2, 208-210], immunomodulators such as IL-2 [2, 211], melanoma tumor antigens, such as MART-1 [2] or gp100 [2], or tumor suppressor genes, such as *p53* [2, 212, 213]. The neoplasias that have been treated so far

with adenoviral-based gene transfer include: melanoma [2], prostate [2, 209], mesothelioma [208, 210, 214], metastatic colorectal carcinoma in the liver [213], lung cancer [212], neuroblastoma [2], glioblastoma [2], ovarian cancer [2], and squamous cell carcinoma of the head and neck [2].

Although adenoviral vector technology has been considerably improved in terms of gene transfer applications, the main issue of vector design remains the avoidance of immune responses. This problem has been tackled from different angles. A number of studies have designed new generations of adenoviral vectors. In this respect, two types of approches have been pursued: deletion of E1, E2 and E4 genes in order to avoid the expression of immunogenic viral proteins within transduced cells [127]; and overexpression via a strong constitutive promoter of the E3-encoded 19 kDa glycoprotein (gp19K) in adenoviral vectors lacking the E1 gene [215, 216]. The latter approach has provided encouraging results in terms of more stable transgene expression in the liver [215, 216] or in the lung of rodents [215]. The function of the E3-encoded gp19K is to inhibit the transport of the major histocompatibility complex class I molecules to the cell membrane [216]. This results in impairment of the antigen-presenting cell mechanism, which avoids the clearance of adenovirally transduced cells by CTL immune responses [217, 218].

Adenoviral vectors lacking E2a function and the E1 gene have allowed for a prolonged but transient, transgene expression in the liver of mice [219], and in correcting the phenotype of ornithine carbamyl-transferase deficiency in a murine model [220]. E2a gene expression was neutralized by mutations that rendered the adenoviral protein temperature-sensitive [219, 221]. The combination of E1 and E4 adenoviral gene deletions has required the construction of helper cell lines to supply the E4 functions [222, 223]. The results that have been obtained in the animal model are rather contradictory. One study has shown significantly longer transgene expression for the double E1 + E4 deletion compared to the single E1-deleted adenoviral vector system [224], if the transgene is not per se immunogenic [225]. Conversely, other studies comparing the double E1 and E4 deletion and the single E1-deleted adenoviral vector systems have either reported no substantial benefit in removing the E4 gene [223], or even a detrimental effect on the duration of the transgene expression for the E1 and E4 deficient system [227]. A variety of factors may be involved in the generation of such contrasting results [127]. One could be the innate immunity of the animal model used in the study [228], or the route of adenoviral vector administration [229], which may also cause the depletion of the adenoviral genome. This underlines the importance of designing and conducting in vivo studies under more uniform conditions in order to avoid dramatic disparities of results among different groups of investigators. However, if minor differences in the modality of administration of adenoviral vectors and genetics of the animal models affect the results of the studies, even greater difficulties should be expected in applying adenoviral vectors in clinical trials. Studies are still in progress to further characterize and evaluate the beneficial role of sequential and combined deletions of early adenoviral genes in terms of suppression of immunogenicity related to adenoviral-mediated gene transfer. Interestingly, recent reports have revealed that open reading frame 3 (ORF3) of the gene E4 is required for the persistent expression in vivo and in vitro of a transgene regulated by an internal CMV promoter [230, 231]. In one study, the adenoviral vector system lacks E1, E2a, E3 and E4 except the ORF3 [231], whereas the other study has addressed the functions of ORF3 and of ORF4, ORF6 and ORF6/7 of E4 gene in the context of an adenoviral vector lacking E1 and E3 [230].

Other strategies have been considered in order to minimize the adverse effects of immune responses related to adenoviral-mediated gene transfer. Basically, these alternative approaches consist of reducing the administration load of the adenoviral vector into the patients, by developing high-efficiency transgene expression vectors combined with short-term immune suppression of the subject [203, 232], and/or by generating chimeric adenoviral capsids, with the intent of enhancing the binding affinity for the target cells [5]. This may be achieved by different means. One possibility is the engineering of an adenoviral vector type 5 carrying the fiber genes of the adenovirus type 7 [233, 234]. This chimeric capsid has an enhanced binding affinity for human lung epithelial cells [233, 234], but it is not suitable for efficient liver-directed gene transfer. An interesting observation is that the fiber swapping between adenovirus type 5 and type 7 may also affect the intracellular trafficking of the adenoviral transfer vector [234]. Another chimera was generated by engineering an adenoviral vector type 2 expressing the fiber gene of the adenovirus type 17, in order to optimize targeting to human airway epithelium [235], which is refractory to the adenovirus serotype 2 infection. It has been demonstrated that efficient adenoviral-mediated gene transfer to the human airway is contingent upon $\alpha V\beta 5$ integrin expression [236]. Another possibility that has been explored is the production of adenoviral vector particles with altered cell tropism, in which exogenous genes are introduced into the adenoviral capsid [237-242]. In one of these studies, the insertion of an Arg-Gly-Asp (RGD) motif into the fiber gene of an adenoviral vector has generated a chimeric vector of expanded cell tropism, and with enhanced transduction efficiency for primary tumor cells [238]. Another study has shown a remarkable increase of adenoviral transduction of muscle cells by fusing in frame a polylysine moiety to the fiber protein of the adenoviral capsid [243]. Other attempts to optimize the adenoviral transduction in vivo consist of combining

adenoviral vectors with nonviral gene delivery systems, such as: lipofectAMINE to enhance the transduction of human T lymphocytes [244] (an analogous approach has already been mentioned for retroviral vector transduction [147]); poloxamer 407 to facilitate percutaneous adenoviral-mediated gene transfer [245]; and polyethylene glycol (PEG)ylation of adenoviral particles to protect them from neutralizing antibodies both in vitro and in vivo systems [246]. The latter study has brilliantly addressed the issue of humoral immune responses to the readministration of adenoviral vectors in the murine model [246]. In this study, the adenoviral capsid has been masked by PEG to prevent the attachment of neutralizing antibodies. PEG has been covalently bound to the capsid via activated tresyl-monomethoxypolyethylene glycol, which preferentially reacts with the amino terminal of lysine residues [246]. These PEG-modified adenoviral vector particles were efficiently protected from humoral immune responses after readministration to the lungs of mice [246]. A recent study has addressed the issue of improving the long-term expression of adenoviral vectors by engineering a hybrid adenoviral/AAV vector system [247]. This approach parallels the previously mentioned adenoviral/retroviral hybrid vector system [113, 114]. The difference is that the adenoviral vector delivers into the animal an AAV-based vector that also can transduce nondividing cells [247].

Other safety issues related to adenoviral-mediated gene transfer are related to the possible formation of a replication-competent adenovirus, and to the previously mentioned dissemination of adenoviral vectors to the gonads, which may potentially result in trasmission to the germ line [56]. A study has raised an interesting point about the possible higher probability of replication-competent adenovirus formation in large scale production of adenoviral vectors [248].

AAV-Based Vector

AAV is a nonenveloped human single-stranded DNA virus that belongs to the genera of the *Parvoviridae* and does not seem to be associated with any human disease [249]. There are five human AAV serotypes. AAV-based vectors are usually based on serotype 1 or 2. They can transduce a wide variety of cells derived from different tissues [250], including nondividing cells [79, 85] and hematopoietic stem cells [251], although a substantial variability in transduction efficiency among different cell lines has been observed. In addition, AAV vectors may allow for stable transgene expression, following integration of the viral genome into the cellular chromosomal DNA [252]. Interestingly, wild-type AAV has the capability of integrating its genome specifically into the q arm of chromosome 19, between q13.3 and qter [80-84]. Unfortunately, this desirable property is not conserved in recombinant AAV vectors [5], raising the problem of possible insertional mutagenesis (Table 1). AAV has a small genome of 4.7 kb. The insertional capacity for foreign genes is limited to the range of about 4 kb. In some studies, transgenes had to be truncated to be adapted to the AAV vector system. The life cycle of AAV consists of two phases: latent infection and lytic phase. Entry into the lytic phase is caused by superinfection of latently infected cells, or by the coinfection of an adenovirus or a herpesvirus [253-255]. Recombinant AAV vector stocks are usually generated by infecting the AAV packaging cell line with an adenovirus. The lytic infection of AAV is triggered by the adenoviral early genes E1 and E4 [256]. Recombinant AAV vectors can be produced at high titers, such as 10^{10} cfu/ml, but it is rather difficult to obtain pure stocks that are helper virus-free. This poses a limitation to the application of AAV-mediated gene transfer in clinical trials. Recombinant AAV vector systems have been engineered following the same routes used for retroviral vectors. The AAV transfer vector is devoid of all the viral genes, except for two inverted terminal repeats (ITRs). The AAV transfer vector may contain either a marker gene or therapeutic factor. Another plasmid contains the AAV *rep* and *cap* genes, which encode for AAV packaging components and are under the control of a constitutive promoter. The ITRs have been removed from this plasmid. The highly transfectable 293 cell line is used as packaging cell line. The AAV transfer vector and the AAV packaging construct are simultaneously cotransfected in 293 cells, which are then infected with adenovirus in order to activate replication of the recombinant AAV vector. The mechanism of viral entry into the target cells has recently been elucidated for AAV type 2 [257, 258]. It was found that AAV type 2 first binds to heparan sulfate proteoglycan [259], and then binds to $\alpha V\beta 5$ integrin [257, 258]. This finding explains the broad cell tropism of AAV, and the variability observed in transduction efficiency among various cell lines, especially for primary human hematopoietic stem cells [260]. Neither low efficiency nor even the failure of transduction of certain target cells are originated by lack of AAV binding to the cell membrane [258]. For instance, the murine NIH3T3 cell line is refractory to AAV infection, despite efficient binding of the virus to the cell membrane via heparan sulfate proteoglycan receptor [258]. Therefore, the differential distribution of $\alpha V\beta 5$ AAV coreceptor among various cell lines accounts for the transduction efficiency variability observed for AAV-mediated gene transfer. The identification of the exact steps of viral entry may have important implications for optimizing gene transfer applications, apart from the prevention of viral infection. An example is the finding that expression of $\alpha V\beta 5$ integrin is an essential requirement for efficient adenoviral-mediated gene transfer to the human airway [236]. To this end, as already mentioned, adenoviral vectors based on serotype 5 have been genetically engineered to

express fibers of serotype 7, which bind to αVβ5 integrin with higher affinity than the fibers of serotype 5 [233, 234]. A study has demonstrated that the capsid of AAV type 2 can also be genetically modified by introduction of short foreign genes [261]. Specifically, a 14-amino-acid peptide containing a RGD motif was inserted into the AAV *cap* gene, in order to confer to the virion an altered cell tropism to infect cells refractory to AAV type 2 infection, such as mouse melanoma B16F10. The transduction efficiency of the chimeric AAV vector was estimated by *LacZ* reporter gene expression in B16F10 cells to be in the range of 10^4 cfu/ml [261]. Another report has generated a gross hybrid system by fusing in frame a single chain antibody with the AAV type 2 capsid to target the CD34 molecule [262]. The transduction efficiency of the chimeric AAV vector was about 10^2 cfu/ml in CD34$^+$ cells [262]. An aspect that is going to be investigated is the low efficiency of recombinant AAV vectors in transducing airway epithelial cells [263]. Epithelial cells express the αVβ5 integrin, which is the coreceptor for AAV type 2. Indeed, the levels of αVβ5 integrin expression allow for an efficient transduction of airway epithelial cells by adenoviral vectors carrying the fibers of serotype 7 [233, 234]. One hypothesis, which may account for the inefficient AAV-mediated gene transfer to airway epithelial cells, is that AAV vectors encounter a physical barrier of negatively charged molecules, such as mucins and glycosaminoglycans [263]. The improvement of AAV-mediated gene transfer to the airway epithelial cells has important implications for the development of gene therapy programs for the treatment of cystic fibrosis. Another study has tackled this problem by systematic administration of recombinant AAV vectors based either on type 2 or type 3 through bronchoscopic delivery in rabbits [264]. The results of this study have indicated that the procedure was safe, as no significant inflammatory responses were observed in rabbits. In addition, the transgene expression was efficient despite the level of neutralizing antibodies to AAV detected in the serum of rabbits [264].

The elucidation of the AAV type 2 entry mechanism into the target cells has been a breakthrough in the field of vector design. Besides providing useful information about how to improve the transduction efficiency for certain cell types, the identification of viral receptors may also be applied for the purification of recombinat AAV vectors, in order to obtain clinical-grade preparations [265].

Nonviral Vectors

Gene delivery systems based on nonviral vectors mainly comprise cationic liposomes [89-92, 272], DNA-protein complexes [93, 94] and mechanic administration of naked DNA [22, 26, 27, 30]. These systems are relatively easy to manipulate. Nonviral vectors are not infectious and are not very toxic. Furthermore, nonviral vectors allow for the delivery of large DNA fragments and are also particularly suitable to deliver oligonucleotides to mammalian cells, which is an excellent feature for the application of antisense strategies to downregulate the expression of certain genes (Table 1). Antisense strategies can be applied to a variety of pathologic conditions, including cancer, infectious diseases, and to prevent the rejection of allograft transplantation of organs [226]. In addition, as already mentioned, liposomes have been used to enhance the gene delivery efficiency of retroviral [147] and adenoviral vectors [244].

A number of obstacles have severely limited the application of nonviral-based vectors in therapy and preclinical studies [5]. The lack of specific targeting, the low transfection efficiency and the fact that transgene expression is only transient make difficult the in vivo applications of nonviral gene delivery systems. In addition, the unmethylated CpG islands of bacterial DNA elicit strong host immune responses [32, 266]. Furthermore, the liposome-mediated delivery of plasmid DNA enhances the immune responses to unmethylated CpG motifs more than the injection of plasmid DNA alone, probably because liposomes increase the cellular uptake of DNA. This is positive for genetic immunization purposes, but it is a considerable drawback to many other in vivo applications. The employment of cationic liposomes for the delivery of plasmid DNA was not succesful in the early gene therapy clinical trials for the treatment of cystic fibrosis, due to inflammatory reactions that were observed in the patients, which are also responsible for the inactivation of the transgene [267]. A recent study has circumvented this issue in an animal model by combining the administration of cationic liposomes with immunosuppressive drugs [268]. Other approaches may be based on the removal and/or methylation of CpG motifs in the plasmids, but this will cause the silencing of the promoter. The utilization of nonviral promoters has also been considered, but the alternative is mammalian housekeeping gene promoters, which are too weak.

Recent studies have reported significant success in improving the in vivo performance of nonviral gene delivery systems [18, 269-271]. These improvements have been achieved by developing new formulations of cationic liposomes, or of other composite nonviral vector systems. Interestingly, one study has generated an oral vaccine that was made of DNA nanoparticles complexed with chitosan, which is a natural biocompatible polysaccharide [18]. The aim of this study was to prevent peanut-induced anaphylaxis in a relevant murine model [18]. This goal was successfully achieved by oral administration of a DNA vaccine encoding for the *Arah2* gene, which is the main peanut allergen. The function of chitosan was to preserve the DNA until it reached the intestine [18]. A similar approach was adopted

in another study in which the plasmid DNA was complexed with atecollagen, a biocompatible polymer [269].

Conclusion

The improvement of vector design has allowed for a broader range of therapeutic applications for gene transfer technology. Gene therapy has a more active role in clinical trials, and there has been a dramatic increase in the number of preclinical studies for gene therapy and genetic immunization programs. However, the degree of vector development is still not sufficiently adequate to meet all the requirements for phase III clinical trials. The field of vector design has to address very difficult tasks from the standpoint of improvement of the transduction efficiency and safety precautions. The assessment of the risk/benefit ratio can be to some extent predicted only for limited cohorts of patients, who usually have poor clinical outcomes and short life expectancy at the time of the enrollment in gene therapy clinical trials. The application of gene-based interventions to other subjects is likely to be obstructed by an unfavorable risk/benefit ratio. In addition, the assessment of the risks associated with administration of viral-based vectors appears more complicated than previously thought. A number of complex issues must be addressed to evaluate the probability of having adverse effects in patients related to the treatment, and to establish the extent of the possible harm that patients may sustain. So far, the safety issues that have been considered are related to the immunogenicity of the vectors, the formation of replication-competent viruses in patients and the presence of contaminating agents in the vector preparations. Other pressing safety issues are related to insertional mutagenesis, possible recombinations between retroviral vectors and HERVs, and transmission of viral and other exogenous DNA to the germ line. To date, it is not easy to assess exactly all these risk factors. The nature of the risks associated with gene therapy treatments must be established and minimized as much as possible, in order to have a more positive risk/benefit ratio in favor of intervention. When all the requirements for more efficient gene delivery and safer therapeutic applications are met, gene transfer technology will become an accepted reality in the clinical setting.

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Gene Transfer Technology in Therapy: Current Applications and Future Goals

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ABSTRACT

Gene therapy has attracted much interest since the first submissions of phase I clinical trials in the early 1990s, for the treatment of inherited genetic diseases. Preliminary results were very encouraging and prompted many investigators to submit protocols for phase I and phase II clinical trials for the treatment of inherited genetic diseases and cancer. The possible application of gene transfer technology to treat AIDS, cardiopathies, and neurologic diseases is under evaluation. Some viral vectors have already been used to deliver HIV-1 subunits to immunize volunteers who are participating in the AIDS vaccine programs in the USA. However, gene delivery systems still need to be optimized in order to achieve effective therapeutic interventions. The purpose of this review is to summarize the latest achievements in improving gene delivery systems, their current application in preclinical studies and in therapy, and the most pressing issues that must be addressed in the area of vector design. ***Stem Cells 1999;17:191-202***

INTRODUCTION

The interest in gene therapy can be dated back to the mid-1960s, well before the advent of recombinant-DNA technology. At that time, the first speculations about the possible treatment of genetic disorders by introducing functional genes via viral-mediated gene transfer had already arisen [1]. This hypothesis became a reality in 1990, with the first phase I gene therapy clinical trial for the treatment of adenosine deaminase (ADA) deficiency [2]. The results were very encouraging. The two young girls who participated in the clinical trial fully recovered from the disease after the treatment and remained asymptomatic, although they are still on enzyme supplementation. This preliminary study can be considered an important event, as it may sanction the advent of gene transfer technology in medicine. This first gene therapy clinical trial was rapidly followed by many others across the USA and worldwide. Between 1989 and 1994, about 100 protocols were approved worldwide for the gene-based therapy of inherited genetic disorders [3]. All these protocols were phase I clinical trials and assessed primarily the degree of toxicity of the various constructs used in the studies rather than evaluating their therapeutic efficiency in patients. The genetic illnesses treated in these phase I clinical trials comprised: ADA deficiency, cystic fibrosis, hemophilia B, alpha-1-antitrypsin deficiency, Fanconi's anemia, Gaucher's disease, Hunter syndrome, and LDL-receptor deficiency.

Also in 1990, the first gene therapy clinical trial for the treatment of patients with melanoma [4] was conducted. The results of this study indicated that retroviral-mediated gene transfer in patients was safe. This finding prompted the submission of many other protocols for gene therapy clinical trials to treat patients affected by cancer, primarily in the area of melanoma [5-10], followed by ovarian carcinoma [11], sarcoma [10], brain tumor [12], and lung cancer [13].

There is also a strong interest in beginning gene therapy clinical trials for the treatment of patients with AIDS, cardiopathies, and neurologic diseases. Indeed, gene transfer technology has already been applied in the phase I and phase II trials for the AIDS vaccine programs, which have recently begun in the USA [14-16]. These vaccine programs are aiming at inducing both humoral and cytotoxic T lymphocyte (CTL) immune responses to HIV-1 in an attempt to eradicate

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the virus from the patients and to develop protective immunity to HIV-1 transmission in healthy individuals who are at risk of infection. In order to elicit CTL immune responses, the viral antigens must be intracellularly processed within target cells to express various peptidic epitopes associated with host HLA class I antigens on the cell membrane. This may be achieved by gene transfer technology, such as viral vectors carrying HIV-1 genes [14-16], or naked DNA [14, 15, 17]. Humoral immune responses are normally directed at the HIV-1 envelope, whereas HIV-1 specific CTL are usually against gag, pol, or nef [18].

To date, the viral vectors used in the AIDS vaccine programs in humans and primates are vaccinia virus and canarypox virus [14]. Other viral vectors based on Semliki Forest virus, rhinovirus, and poliovirus are currently under development [14]. Vaccinia viral vector has been engineered to deliver HIV-1 envelope (gp120 or gp160) together with the p24 subunit of gag (gag p24) [14], whereas the canarypox-based viral vector has been used to deliver only gag p24 [14]. Subunits of pol and nef have not been tested yet.

There is an enormous variety of possible applications of gene transfer in therapy.

Hopefully, this innovative HIV-1 vaccine design will overcome the complex issue of viral diversity, which, besides posing a key obstacle to the development of vaccines to HIV-1 [19], displays a fundamental role in the pathogenesis of AIDS [20, 21].

There is an enormous variety of possible applications of gene transfer in therapy. As already anticipated, the spectrum ranges from the treatment of inherited or acquired genetic disorders to cancer, AIDS, cardiopathies, and neurologic diseases. This is strongly encouraging to the pursuit of gene therapy programs in medicine. However, after a first phase of enthusiastic research developments, the expectations of investigators are now more sober. Although much effort has been directed in the last decade toward improvement of protocols in human gene therapy, and in spite of many considerable achievements in basic research, the therapeutic applications of gene transfer technology still remain mostly theoretical. The weakest point of gene therapy development programs is, paradoxically, vector design, followed by gene regulation and avoidance of immune responses. Basic research is cautiously progressing to address these pressing issues. The goal of this review is to summarize the standpoint of the various basic research projects, which have been planned to improve the protocols of oligonucleotide and gene delivery in therapy.

Gene Transfer Models

There is a wide variety of vectors used to deliver DNA or oligonucleotides into mammalian cells, either in vitro or in vivo. The most common vector systems are based on retroviruses [22-26], adeno-associated virus (AAV) [27-36], adenovirus [37-45], herpes simplex virus (HSV) [46], cationic liposomes [47-50], and receptor-mediated polylysine-DNA complexes [51, 52].

Other viral vectors that are currently under development are based on lentiviruses [53-58], human cytomegalovirus (CMV) [59], Epstein-Barr virus (EBV) [60], poxviruses [61, 62], negative-strand RNA viruses (influenza virus) [63], alphaviruses [64], and herpesvirus saimiri [65]. Also of extreme interest is the construction of a hybrid adenoviral/retroviral vector, which has successfully been used for in vivo gene transduction [66]. The characteristics of the most developed gene delivery systems are summarized in Table 1.

The stage of development of vectors and their variety are still not sufficient to be efficiently applied in therapy. The treatment of each disease requires specific vector design. For instance, the property of retroviruses to infect only dividing cells [67] is desirable for the selective targeting of neoplastic cells over normal tissues, but it makes retroviruses unsuitable for the transduction of terminally differentiated cells, such as neurons and myocytes. This, of course, rules out the employment of retroviral vectors for the treatment of neurologic and cardiac diseases. On the other hand, viral vectors capable of infecting nondividing cells (adenovirus, AAV, and lentiviruses) may not be suitable for in vivo administrations in cancer therapy because of the side effects that can be originated by the lack of discrimination between neoplastic and normal cells, which, inevitably, will lead to the ectopic expression of the transgene in normal tissues.

The difficult tasks of vector design have to deal with safety issues, improvement of in vivo gene delivery efficiency, and gene regulation post-cell transduction. These tasks are all related to one another. Most of the previously mentioned phase I gene therapy clinical trials for the treatment of inherited genetic diseases and cancer were carried out by ex vivo administration of retroviral vectors into target cells, which were then reimplanted into the patients (i.e., treatment of ADA-deficiency, hemophilia B, Fanconi's anemia, Gaucher's disease, Hunter syndrome, LDL-deficiency, and melanoma). In contrast, the treatment of cystic fibrosis was carried out by in vivo administration of vectors based on adenovirus, cationic liposomes, or AAV. The parameters

Table 1. Description of the main gene delivery systems

Vectors	Characteristics	Disadvantages
Retroviruses	Relatively high titers (10^6-10^7 cfu/ml).	Random insertion of viral genome, which may possibly result in mutagenesis.
	Broad cell tropism.	
	Stable gene expression.	Possibility of replication competent virus formation by homologous recombination.
	No toxic effect on infected cells.	
	Total insert capacity in the virion is in the range of 10 kb.	
	They only infect dividing cells.	
Lentiviruses	They can infect nondividing cells.	Serum conversion to HIV-1.
	They can be pseudotyped with retroviral or VSV G envelopes. therefore, they also have broad cell tropism.	Possible proviral insertional mutagenesis in target cells.
	Stable gene expression.	Presence of tat and rev regulatory proteins, (the early lentiviral vectors also have some HIV-1 accessory proteins).
	Total insert capacity in the virion is in the range of 10kb.	
Adenoviruses	Very high titers (10^{10} pfu/ml).	Host immune response.
	Transiently high levels of gene expression.	Not suitable for long-term expression due to the lack of integration into host genome.
	They can also infect non-dividing cells.	
	Large DNA inserts can be accomodated in the vector (7-8 kb).	Complicated vector genome.
Adeno-associated viruses (AAV)	Wide range of cells can be infected. including cells which do not divide.	High titers of pure virus are difficult to obtain.
	Ability of the virus to establish latent infection by viral genome integration into cell genome.	This vector system is still not well characterized.
		Limited capacity for foreign genes (about 4 kb).
	Viral integration specific for human chromosome 19 (only for wild-type AAV).	AAV requires a helper adeno- or herpesvirus for replication.
	Nonpathogenic, nontoxic.	
	Small genome (5 kb).	Lack of specific integration for recombinant AAV vectors.
Cationic liposomes	They are not infectious.	Targeting is not specific.
	Theoretically, there is no limit to the size of DNA.	Low transfection efficiency.
		Only transient expression.
	Low degree of toxicity.	Diffult in vivo applications.

of these in vivo administrations of vectors in clinical trials are still far from ensuring efficient therapeutic interventions. The vectors used in these studies had some positive properties and were relatively safe. As summarized in Table 1, these gene delivery systems can transduce nondividing cells, avoid cell mutagenesis due to the random transgene integration in the host chromosomal DNA (except for AAV-based vectors) and can be rather easily administered to the patients in high doses; however, they are affected by many limitations. Adenoviral vectors can elicit host immune responses and are not suitable for long-term expression of the transgene, especially in vivo. Liposome-based vectors are not infectious and have a low degree of toxicity, but they also do not allow for stable transgene expression, and their in vivo applications are difficult for a variety of reasons (Table 1). The interest in AAV is mainly related to its property of integrating the viral genome in a safe host chromosomal site [31-35]. Unfortunately, such a property is lost in AAV recombinant vectors, and this may result in cell mutagenesis.

The field of gene therapy is now actively involved in the challenging task of improving the design of vector systems for in vivo applications.

Vector Design for In Vivo Gene Delivery

The ex vivo gene delivery approach is certainly a safer procedure than the in vivo one, but poses several limitations to possible gene therapy interventions. The ex vivo approach can obviously be applied only in a restricted number of diseases, as it is a complex process that requires the surgical removal of certain cell types, followed by the in vitro cell transduction and reimplantation into the host. All these manipulations are costly for the health care systems, cause distress to the patients, and cannot always be performed. Conversely, in vivo gene delivery can be easily adapted to the treatment of every disease; it does not particularly distress the patients, as the intervention is not invasive; and it is more affordable. However, the improvement of in vivo gene delivery protocols involves many complicated issues that the field of gene therapy is currently trying to address. For the moment, the strategies of basic research seem to be mainly polarized by

viral vectors based on retroviruses, lentiviruses, AAV, and adenoviruses, in order to develop optimized vector design for in vivo gene transfer protocols. Liposome-based vectors are particularly useful to deliver oligonucleotides or large-size transgenes, but unfortunately, their in vivo applications are difficult.

Each vector system has a series of advantages, problems, and preferential applications in therapy. As previously mentioned, the problems in vector design for in vivo applications are generally related to safety issues, improvement of vector production, and control of transgene expression post-cell transduction. The first rule in the matter of vector design is that the gene delivery systems must not be pathogenic or toxic to the patients. Therefore, the various viral vectors must be engineered to be noncompetent for replication and must not contain viral genes encoding for factors which may pose a hazard in humans. It has been argued whether the removal of putative virulence may be detrimental to the transduction potential. Results indicate that viral vectors so far produced retain their infectivity, although they do not replicate.

Retroviruses are among the most efficient tools for gene transduction of mammalian cells.

The in vivo administration of viral vectors requires additional safety regulations compared to the ex vivo one. In order to avoid the ectopic expression of the transgene, viral vectors should be engineered to have a cell tropism specific for the target cells, especially if the viral vectors can also transduce nondividing cells. In this respect, there have been many attempts, with small success, to alter the cell tropism of viruses that are nonpathogenic in humans in order to engineer chimeric viruses capable of infecting distinct human cells. These studies involved mainly recombinant retroviruses and lentiviruses and will be described in the next paragraph.

Another line of investigation is aiming at controlling in vivo transgene expression by developing vector systems containing internal tissue-specific or inducible promoters. The latter are based on: metalloprotein gene promoter, steroid or tetracycline-inducible promoters. Cre/LoxP recombination system, promoters responsive to the insect hormone ecdysone and retinoids. The in vivo regulation of transgene expression within the therapeutic window is also a very important goal that must be achieved. Unfortunately, there are many elusive problems to be solved which derive mostly from the empirical knowledge basic researchers have in this matter.

The site-specific proviral integration in the host chromosomal DNA is another strongly desired feature. Possibly, this may be accomplished by opportune rearrangement of AAV-based vectors.

Other issues that vector designers are dealing with are: avoidance of immune responses (in the case of adenoviral vectors), improvement of high-titer viral vector stocks, and purification procedures.

Some progress has been made in improving the various gene delivery systems. Their variety is too vast to be described in greater detail, therefore, only the main vector models will be reviewed.

Retroviral and Lentiviral Vectors

Undoubtedly, retroviruses are among the most efficient tools for gene transduction of mammalian cells. For this reason, they were successfully used in the early gene therapy clinical trials for the treatment of inherited genetic diseases [2, 3] and cancer [4-13]. The most common retroviral vector is based on the amphotropic Moloney murine leukemia virus (MLV) [68]. This system is particularly suitable for efficient in vitro cell transduction: the amphotropic MLV has a broad cell tropism, it can be produced at relatively high titers (10^6-10^7 iu/ml), and allows for long-term transgene expression because of the viral integration in the host chromosomal DNA.

Another important feature of retroviruses is that although they do not elicit immune responses in the host, they are susceptible to rapid degradation by the complement [69]. This is a major limitation for in vivo retroviral-mediated gene transfer. Optimal titers for in vivo applications should be in the range of 10^{10} iu/ml, whereas the maximum titer that can be obtained barely reaches 10^7 iu/ml. In addition, retroviral particles are difficult to concentrate, as they are fragile and can be destroyed during the precipitation. This problem can be circumvented by pseudotyping the retroviral core with the G glycoprotein of vesicular stomatitis virus (VSV G). This envelope stabilizes the retroviral particles, which can then be easily concentrated by ultracentrifugation of the retroviral supernatant [70, 71].

Retroviral stocks are mainly produced by transient expression systems [72-76], which offer a variety of advantages: the retroviral titers are in the range of 10^6-10^7 iu/ml, that are from 10- to 50-fold greater than those obtained by conventional packaging cell lines; the production of retroviral stocks is rapid and highly reproducible; the transient retroviral expression practically rules out the possibility of replication-competent virus formation. The latter feature may greatly facilitate the in vivo retroviral-mediated gene transfer.

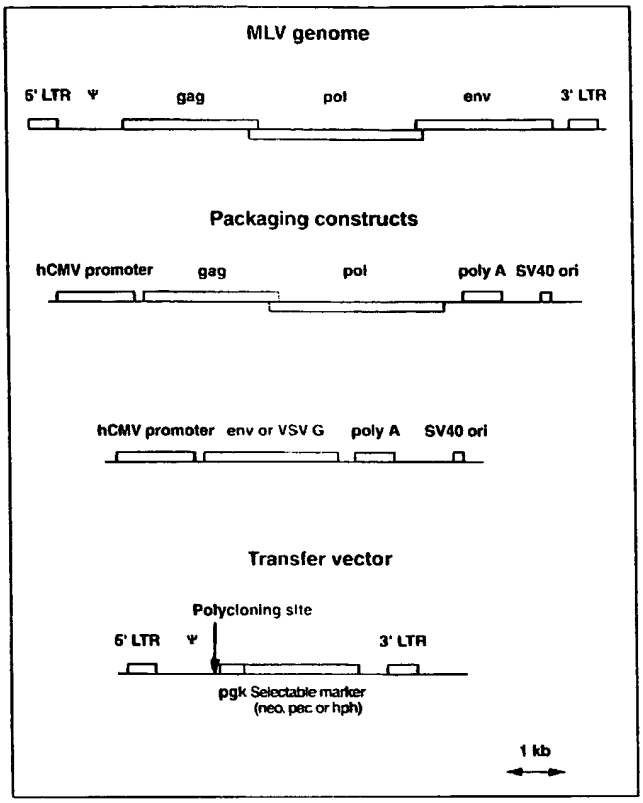

Figure 1. Murine leukemia virus (MLV)-based retroviral vector system. *Abbreviations: pgk = murine internal promoter driving the expression of a selectable marker; neo = neomycin, puc = puromycin; hph = hygromycin.*

As shown in Figure 1, the retroviral genome was divided among three plasmids. Both gag/pol and the envelope (env) are under the control of the human cytomegalovirus (CMV) promoter. The 5′ and 3′ long-terminal repeats (LTRs) and the packaging signal (Ψ) were deleted in these two constructs, therefore, the mRNA encoding for gag/pol and for env is the only substrate for translation in the transfected cells. The retroviral-transfer vector has the two LTRs and the packaging signal (Ψ) and encodes for a chimeric gene whose mRNA can be packaged into the virion and reverse-transcribed in the target cells' cytoplasm; the resulting cDNA is then delivered to the cell nucleus and integrated into the host genome. The chimeric gene may be a therapeutic factor and/or a reporter gene. The production of high-titer retroviral stocks is carried out by transient cotransfection of the three plasmids (gag/pol, env, and transfer vector) in highly transfectable cell lines that express the SV40 large T antigen [73]. The plasmids containing the gag/pol and env cassettes carry the SV40 origin of replication in their backbone. Therefore, post-cell cotransfection, the plasmids' copy number is greatly enhanced by the SV40 large T antigen [75]. The high DNA copy number and the massive production of gag/pol and env by the strong human CMV promoter result in an optimized retroviral titer [73, 75]. The recombinant retroviral vector was engineered to sustain a single round of infection, and the fact that the proviral genome was divided among three plasmids rules out the possibility of replication-competent virus formation by homologous recombination [75].

Also of interest is the production of new retroviral transfer vectors, which were genetically engineered to maximize the transgene expression post-cell transduction, especially in cells of hematopoietic origin [77]. In these transfer vectors, the LTRs have been modified by point mutations to increase transcription activity post-viral integration in the host genome [77]. This feature is meant to improve the performance of retroviral vectors in preclinical in vivo studies and, possibly, in therapy.

Retroviral transfer vectors have also been designed to deliver transgenes under the control of internal inducible or tissue-specific promoters [78, 79]. The presence of an extra internal promoter may interfere with the 5′ LTR transcriptional activity, and/or vice versa [79]. For this reason, the retroviral vectors were engineered to have an active 5′ LTR in the proviral form, which is then disactivated after the viral genome integration in the host chromosomal DNA. This may easily be achieved by performing a small deletion in the 3′ LTR of the proviral transfer vector [78]. Such retroviral vectors have been named self-inactivated vectors (SIV) [78].

Another important line of investigation is considering the engineering of chimeric retroviruses with specific cell tropism. This would greatly facilitate the in vivo application of retroviral vectors in clinical trials. In this respect, there have been many attempts to alter the cell tropism of ecotropic retroviruses, which do not infect human cells. This approach consists of placing foreign genes into the retroviral envelope in order to confer a cell tropism specific for certain human cell types. The foreign genes used in the early studies to generate hybrid envelopes were: CD4 [80, 81], single-chain antibodies [82-84], the polypeptide erythropoietin [85], short peptides binding to several integrins [86], and human heregulin [87]. The retroviral systems used in these studies were: avian leukosis virus [80, 86], ecotropic MLV [81, 82, 85, 87], spleen necrosis virus [83, 84], and amphotropic MLV [88]. In some cases, there has been a partial success in redirecting the cell tropism of ecotropic retroviruses [81, 83-88], but the transduction efficiency is far from being optimal for in vivo applications. A number of more recent reports have shown some improvement of transduction efficiency by chimeric viral particles with altered cell tropism [89-91]. The viral vectors used in these studies were based on adenovirus [89, 90] and on Sindbis virus [91]. Interestingly, two other groups of investigators have engineered chimeric rabies virus [92] and VSV

[93], which were pseudotyped with CD4- and CXCR4-derived proteins. The latter is the coreceptor for T cell tropic HIV-1 strains [94, 95]. These studies showed that both chimeric viruses selectively infected and induced cytopathic effects in cultured cells harboring HIV-1 [92, 93]. This finding is certainly a leap forward from the preliminary study conducted by *Young et al.* [80]. However, it remains to be confirmed whether these chimeric viruses will be able to seek out and selectively destroy HIV-1 infected cells in the in vivo model.

An important property of retroviruses is that they can only infect actively dividing cells [67], as the transport of the preintegration complex to the nucleoplasm requires the breakdown of the nuclear membrane. Conversely, lentiviruses, such as HIV-1, also have the capability of infecting nondividing cells [96-98]. The requirement for cell division for retroviral infection has relevant implications in gene transfer technology. A positive aspect is that in vivo retroviral-mediated gene delivery in cancer therapy is facilitated because of the specific gene targeting of neoplastic cells over normal tissues. On the other hand, the lack of retroviral infection of nondividing cells precludes their in vivo gene transfer applications for neurons, hepatocytes, myofibers, and hematopoietic cells. In this prospective, the engineering of HIV-based lentiviral vectors will prove very useful. Many nonproliferating cell lines can be easily manipulated with this HIV-based vector system to generate cell culture models that stably express transduced genes. Preliminary in vitro experiments indicated that terminal differentiated neurons [99] and terminal differentiated macrophages [57] were efficiently transduced, and the reporter gene expression was stable. This finding mirrors that of another in vivo study, in which a lentiviral vector carrying a reporter gene was injected into adult rats' brains, in order to transduce neurons [53, 54]. In this case too, efficient gene delivery and a stable expression of the transgene were observed. The lentiviral-based vector systems are most likely going to implement the therapeutic efficiency of gene transfer technology in the near future. Before then, the lentiviral vectors must be thoroughly tested for biological safety. The possible reconstitution of pathogenic replication-competent HIV-1 must be excluded. The lentiviral vector stocks are also generated by transient overexpression systems [73-76], in which the packaging components (gag/pol and env) have been placed on two different plasmids and are under the control of the human CMV promoter, and the transfer vector is on a third plasmid [53]. Furthermore, the HIV-1 envelope has been deleted in this system, to be replaced by the amphotropic MLV or VSV G envelopes [53]. The HIV-1 genome has six additional reading frames to the prototypic gag, pol, and env genes that are common to all retroviruses (Fig. 2). These extra six reading frames encode for the following factors: tat, rev, vif, vpr, vpu, and nef. Viral replication is mediated by the so-called regulatory tat and rev proteins, which respectively control viral transcriptional and post-transcriptional pathways. The other four factors (vif, vpr, vpu and nef) are called "accessory proteins" [16]. The function of these accessory proteins in HIV-1 pathogenicity is very complex and not completely understood. They are essential to maintain virulence in vivo [100] and may interfere with the cell cycle and/or cell growth [16, 100]. Their presence may per se represent a hazard in humans, regardless of the lack of HIV-1 infection. In two latest reports, the accessory proteins were deleted from the lentiviral vector system without compromising their transduction efficiency [57, 58]. This is another substantial step forward in the development of a safer lentiviral vector system. There are still many other aspects of lentiviral's biology that have to be investigated prior to considering their application as vectors in clinical trials. The

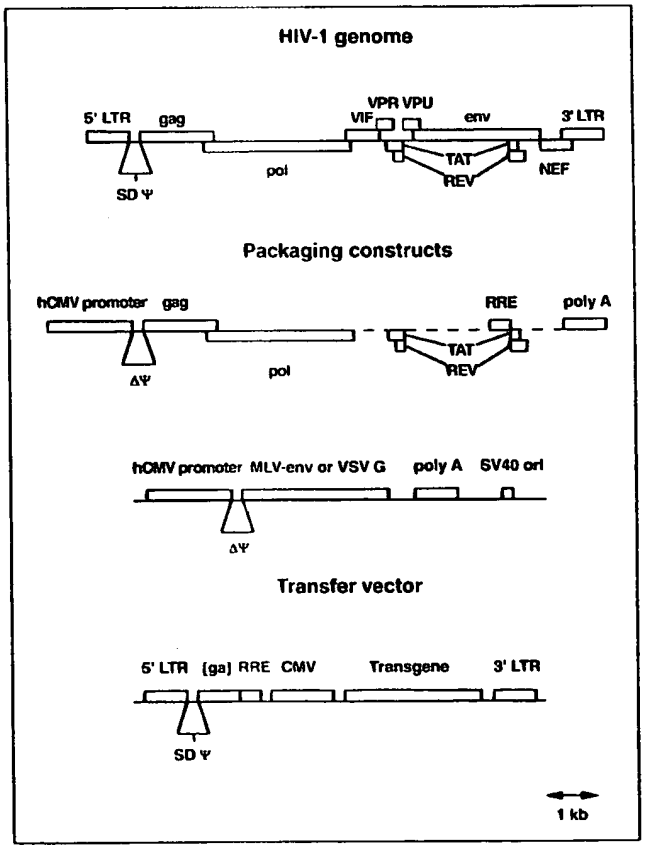

Figure 2. Lentiviral vector system. *Abbreviations: SD = splicing donor site; RRE = rev response element, [ga] = initial fragment of gag. The dashed line reported in the first packaging construct indicates the deletions that have been made in the HIV-1 genome.*

main concern is about possible cell cycle and/or cell growth dysregulations by tat protein, and the random proviral integration in the host genome, which may result in mutagenesis. This phenomenon may be more dramatic for in vivo applications of lentiviral vectors than for retroviral-mediated gene transfer because of the capability of lentiviruses to also infect nondividing cells. This may predispose the lentiviral-based vectors in delivering and inserting the transgene into the genome of wrong cell types or tissues, provoking possible harm to the patients.

The design of HIV-based vectors is still very demanding in terms of biosafety regulations. On these grounds, it is not easy to predict whether and when this vector system will be used in gene therapy clinical trials.

Adenoviral Vectors

Adenoviruses, together with retroviruses, constitute the most advanced gene therapy forefront of the basic research development for gene delivery systems.

Adenoviruses are large non-enveloped DNA viruses with a double stranded genome of 36 kb and a capsid diameter ranging from 65 nm to 80 nm [38, 39]. So far, 49 serotypes of human adenoviruses have been identified and classified into six groups according to similarities in their genome organization and hemagglutinin activity. The diameter of the viral particles depends on the serotype. Human adenovirus was isolated for the first time in 1953, when a spontaneous in vitro culture degeneration of some adenoidal tissues was observed [37]. Later, it was found that the etiologic agent responsible for this cytopathic effect was a virus, which was the reason for its being named "adenovirus" [101]. The various adenoviral serotypes can be found in distinct tissues, such as the upper respiratory tract, the conjunctiva, and the intestines.

The first recombinant adenoviral vectors were engineered in 1985 and were based on the serotypes 2 and 5 [40-42]; they are not associated with severe diseases and do not cause tumors in animals, in contrast to the other serotypes. The first adenoviral-mediated gene transfer applications in clinical trials were carried out at the beginning of the 1990s for the treatment of patients affected by cystic fibrosis [102]. Probably, adenoviral vectors will also be employed soon in cancer therapy and in the treatment of familial hypercholesterolemia and neurological and cardiovascular disorders. Many in vitro and in vivo studies in animal models have already been performed along these lines of research [103-107]. As anticipated, adenoviruses are highly immunogenic and may originate inflammatory and toxic reactions in the host [108, 109]. This poses a severe limitation to the possible applications of adenoviral-mediated gene transfer for the treatment of hereditary disorders, cardiopathies, and neurologic diseases. In addition, in all these illnesses, long-term transgene expression is required. Adenoviral vectors only allow for transient expression, because the adenoviral genome is extrachromosomal in the infected cell.

On the other hand, adenoviral-mediated gene transfer offers some advantages over retroviral vectors. First of all, adenoviral vectors can be produced at very high titers (10^{10} pfu/ml), which can be easily concentrated to 10^{12} pfu/ml. The adenovirus has the capability of encapsulating DNA molecules up to 6% bigger than the wild-type viral genome; therefore, 7-8 kb DNA inserts can be introduced in the vector. Theoretically, it may be possible to introduce in the virion much bigger DNA fragments than 7-8 kb, providing that the adenovirus genome is properly deleted. Adenoviruses can also infect nondividing cells, in contrast to retroviruses. Adenoviral-mediated gene transfer allows for high transient overexpression of the transduced gene.

The improvement of adenoviral vector design has to deal with the problem of immunogenicity. Most likely, the leaky E2 gene expression of the adenoviral vector system is responsible for the toxicity and inflammatory reactions. Studies are currently in progress to design new generations of adenoviral vectors lacking E2a-gene functions, either by mutations [110, 111] or by deletion of E4 genes, which requires the construction of helper cell lines that can provide E4-function [112, 113].

Other strategies that are currently pursued to avoid immune responses are directed at reducing viral load by developing high-efficiency transgene expression vectors in combination with short-term immune suppression [114, 115] and/or by generating chimeric adenoviruses type 5 carrying fiber genes of adenovirus type 7 [116]. The advantage of using such a chimeric capsid is the binding affinity enhancement of the adenoviral particle to the target cell.

Adenoviral/Retroviral Chimeric Vectors

A chimeric adenoviral/retroviral vector system has recently been developed [66] in order to combine the advantages of adenoviruses and those of retroviruses in a single gene transfer system. This may allow for the simultaneous achievement of more efficient gene delivery and longer-term transgene expression. Both features are necessary to optimize the in vivo therapeutic gene transfer interventions to correct human defective genes. Briefly, this gene delivery system consists of an adenoviral vector carrying in its genome the packaging components of a retrovirus together with the retroviral transfer vector, which is the recipient for transgenes. As already mentioned, the adenoviral vector can be produced at

very high titers and can also infect nondividing cells. The adenoviral genome is transiently overexpressed in transduced cells, as it is not integrated into the host genome. At this stage, the transduced cells produce retroviral vectors capable of infecting other surrounding cells. This may improve the efficiency of in vivo retroviral transduction. Once certain tissues have been infected by the chimeric adenoviral/retroviral vector system, retroviral vectors are produced in vivo over a considerable period of time and can reach their target cells. The constitutive localized production of retroviral vectors may, at least partially, overcome the complex issue of complement-mediated lysis of retroviral particles that occurs in the in vivo model. However, this system needs to be improved and better characterized before it can be applied in clinical trials; the immunogenicity of adenoviral vectors must be completely *devoided*; there is still the possibility of proviral insertional cell mutagenesis; the retroviral titers are still too low for effective in vivo applications.

AAV-Based Vectors

AAV is a human parvovirus that does not seem to be associated with any human disease [27]; therefore, the first requirement for gene therapy applications is easily accomplished. In addition, AAV has many desirable properties: it can infect a wide range of cells deriving from different tissues [28]; it can also infect nondividing cells [30, 117]; it can establish a latent infection by integrating its genome [29]; the integration of the viral genome is site-specific for the q arm of chromosome 19, between q13.3 and qter [31-35]. All of these properties explain the considerable interest in applying AAV as a vector in gene therapy. The site-specific integration of AAV is a desired safety feature that is, however, lost in AAV recombinant vectors. The major research aim is to conserve the site-specific integration of AAV vector systems, possibly by cotransfecting a plasmid encoding the protein Rep78, which seems to be responsible for the viral-specific integration process in the presence of the inverted terminal repeats [118, 119]. Other problems for the application of AAV-based vector systems are related to the limited capacity of accommodating foreign genes, that is, those in the range of 4.1-4.9 kb [120]; to the difficulty of obtaining pure high-viral titers, and the requirement for helper adeno- or herpesvirus for replication in cell culture [121-123]. The inability to completely eliminate helper viruses has raised an element of concern about the application of AAV vectors in clinical trials.

In preliminary experiments, recombinant AAV vectors have stably transduced a certain number of nondividing cells, such as hematopoietic progenitor cells [124], neurons [125], and photoreceptor cells [126]. Another encouraging finding is the lack of immune response to in vivo AAV-mediated-gene transfer [127]. It is likely that recombinant AAV vectors will be employed for the treatment of cystic fibrosis [128] instead of adenoviruses.

Cationic Liposomes and Other Nonviral Vector Systems

Nonviral vector systems comprise various formulations of cationic liposomes [129-131] and composite vectors devised for gene delivery applications by receptor-mediated entry containing a DNA-binding moiety, a receptor-targeting molecule, and often a lysosome-breaking agent [132-135].

These gene delivery systems are not infectious and have a low toxicity. Theoretically, there is no limit to the DNA size that liposome particles can carry. Furthermore, liposome-based vector systems are suitable for the delivery of oligonucleotides to mammalian cells. Receptor-mediated gene delivery systems have the additional advantage of a potentially specific target. The disadvantages of both systems are low transfection efficiency and the transiency of gene expression. Cationic liposomes have the additional disadvantage of lack of specific targeting, whereas receptor-mediated delivery systems may be immunogenic.

Cationic liposomes have already been employed in phase I clinical trials for the treatment of cystic fibrosis [136].

Conclusion

The interest in gene therapy is motivated by a variety of reasons. The early successes of phase I clinical trials for the treatment of inherited genetic diseases and cancer have strongly encouraged worldwide establishment of gene therapy research programs, which are also evaluating the possibility of treating patients with AIDS, cardiopathies, and neurologic diseases. In addition, gene transfer technology has led to innovative vaccine design for the treatment of neoplasias and development of protective immunity against infectious agents. Studies are currently in progress to find vaccines for malaria and Ebola, whereas phase I and phase II clinical trials for the AIDS vaccine programs have already begun in the U.S.

The standpoint of gene therapy basic research is still far from providing the tools for the treatment of the previously mentioned illnesses. The most pressing issue that the field of gene therapy has to address is the development of efficient in vivo gene delivery systems. The in vivo administration of either functional genes or therapeutic factors would greatly simplify and improve any human gene therapy intervention.

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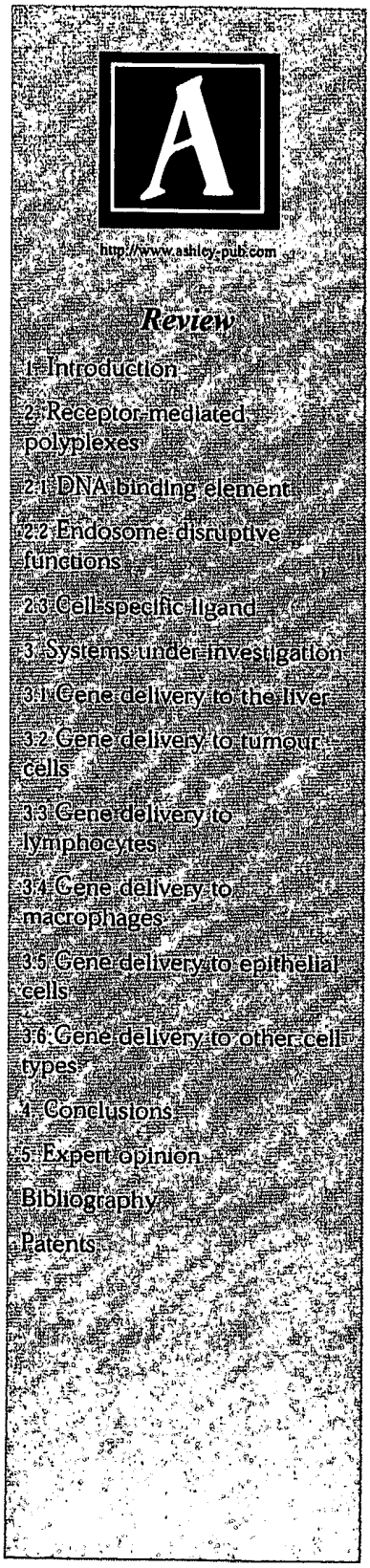
Expert Opinion on Therapeutic Patents



Ligand-targeted receptor-mediated vectors for gene delivery

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Gene therapy promises to cure human genetic diseases. One of the main obstacles to fulfilling this promise is in the ability to target a gene to a significant population of cells and express it at adequate levels for a long enough period of time. Viral methods for gene delivery have been studied for a number of years and are effective vectors for gene transfer. The great majority of gene therapy clinical trials currently in progress use retroviruses or adenoviruses. However, there are concerns for their clinical use because of possible risks of mutagenesis, immunogenic side-effects and toxicity. In addition to this, there are other limitations, including the size of gene that can be transferred. Over the last ten years, a new approach has emerged that has increasingly gathered speed thanks to advances in receptor cell biology and antibody production. This method involves ligand-targeted receptor mediated endocytosis (RME) of 'polyplexes'. Here, synthetic complexes are composed of a cell-specific targeting ligand, coupled to a DNA binding element and endosmolytic function. These complexes are able to deliver genes to cells in a receptor-specific manner, without any viral DNA sequences or packaging constraints. There are now many ligand/receptor systems under investigation, each one demonstrating successful gene transfer with a higher level of tissue specificity than viruses can offer. This review describes most of these systems and looks ahead to an era where cell-specific gene delivery may be a main stream gene therapy, treatment modality.

Keywords: *polyplexes, receptor-mediated endocytosis, targeted gene delivery, vectors*

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1. Introduction

There are few areas of biomedical research that have moved as rapidly or so completely captured the imagination of the scientific community as the field of gene therapy. Although in its infancy, gene therapy is a huge commercial business judging by the number of patents filed and of small biotechnology companies starting up, based upon one particular gene therapy technology. This phenomenon surely reflects the enormous potential of this approach for the correction of genetic diseases. There is certainly no lack of ideas, although many are hampered to an extent by the limitations of current technologies. General approaches include replacing a defective gene in diseases such as cystic fibrosis or muscular dystrophy, destroying cancerous or virally-infected cells such as in HIV infection or by promoting a host immune reaction against a disease or infection (for reviews see [1,2]).

When antibodies were discovered, a similar wave of optimism followed. Early this century, Ehrlich hypothesised about 'magic bullets', able to destroy target cells on a specific basis. Monoclonal antibodies brought us a step closer to this dream. Since then, there has been a great deal of research in the area of antibody- or ligand-mediated delivery of drugs, toxins, radioactive isotopes and enzymes, with many promising leads entering clinical trials. Protein engineering has allowed some of these molecules to be improved, and this area is currently one of the most exploited in the biotechnological industry. However, even after almost 30 years of relentless pursuit, nothing has yet delivered such a promise in terms of clinical results.

The delivery of genes encoding various functions greatly expands the range of treatable diseases as well as the types of strategies which can be employed. However, gene delivery remains the major technological stumbling block in gene therapy strategies [3]. Viruses are well suited to deliver genes to mammalian cells by virtue of their infection and replication cycle. Viral delivery is by far the most commonly utilised form of gene transfer vector (reviewed in [4]), with retroviruses being used for many years. Over the last few years, adenoviruses have been developed to overcome some of the limitations of retroviruses and, recently, the two types of viruses have been 'married' to produce a hybrid virus which is able to carry out some of both functions [5]. Retroviruses have the advantages of being potentially low in immunogenicity, with the ability to infect and deliver genes to dividing cells, integrating randomly into the host genome allowing long-term gene expression and heritable transfer. Adenoviruses are able to infect quiescent and dividing cells with much higher efficiency but, being non-integrative, cannot be maintained for long periods of time. They can also provoke a damaging immune reaction.

Whichever viral vector system you chose there are many other drawbacks:

- the risk of secondary malignancies (oncogene activation or tumour suppressor gene disruption) from integrating vectors
- the recombination of disabled viruses could make them infective again
- there is no specific cell specificity, thus allowing non-targeted cells to be infected, a problem compounded by the heterogeneity of virally targeted antigens
- retroviruses cannot infect non-dividing cells
- there is an inherent difficulty in producing high titres of retroviruses for clinical use, although there are some strategies being developed to overcome this
- inactivation by host complement (a natural response to viral infections) in the cases where the infected cells are required to survive, expression of toxic viral genes or the immunogenic response to cells infected can limit the actual number of cells transfected

Finally, one of the greatest limitations of viral gene delivery is in the permitable size of the packaged DNA [6].

There is, of course, a great deal of research aimed at developing viral vectors with improved attributes, such as the recently developed lentivirus vectors (such as HIV), which can infect non-dividing T-cells [7] and retroviruses [8] or adenoviruses [9] which express targeting ligands on their surfaces. However, there is also a growing body of research into alternative, non-infectious gene delivery methods (reviewed in [10]). The main examples of these are:

- liposomes (lipid encapsulated DNA) which fuse directly with cells to introduce their DNA
- naked DNA (cost-effective injection of pure DNA into sites of the body receptive to DNA uptake)
- ligand-targeted receptor-mediated endocytosis of polyplexes

The latter of these is an intensely studied and emerging area and is the subject of this review. Liposomes, like viruses are non-targeted and can cause host complement depletion. Their lipophilic nature gives them the ability to transfer DNA to cells with high efficiency. Naked DNA is simple but non-targetable and suffers from low levels of gene expression and transduction efficiencies. RME of DNA by ligands exploits the highly efficient internalisation pathway and trafficking routes within cells (as do viruses). Clustered ligand/receptor complexes gain entry into the cell by membrane invagination into clatherin-coated pits to form endosomes. Various intracellular trafficking events result in the release of the ligand inside the cytosol with some receptors recycling back to the cell

surface and some being destroyed by lysosomal degradation (for an example, see [11]).

The initial concept of gene delivery by a non-viral internalised ligand was proposed by Cheng *et al.* [12], but this group was unable to report successful gene expression. Wu and co-workers [13] exploited the well-studied, liver-specific ASGP-R to successfully deliver and express genes which were attached to one of its natural ligands, asialorosomucoid (ASOR). In effect, the ligand/receptor pathway was being 'hijacked' into additionally transferring a gene. By such routes, thousands of ligands are internalised per second, hence many gene copies can be targeted to cells. Some of these ligand/receptor complexes are highly specific to certain cell-types, opening up the attractive area of *tissue-specific* targeting of genes. Even so, this is not an entirely new delivery pathway for molecules as this has been the primary route for delivering toxins to tumour cells, exploiting the many tumour-associated antigens which are internalised [14]. For gene delivery, the targeting moiety takes the form of an antibody, peptide or natural ligand and the DNA is attached through a DNA binding agent, usually a polycation such as poly-L-lysine, which serves to complex and compact the DNA. These types of gene delivery vectors have been designated 'molecular conjugate vectors' or 'receptor-mediated gene transfer complexes'. It has now been generally accepted that they should be called receptor-mediated 'polyplexes' [15].

2. Receptor-mediated polyplexes

The most basic vector takes the form of a cell-specific ligand and a DNA coupling element. Various elements, such as whole, disabled adenovirus particles, membrane active peptides or translocation domains (see below), have additionally been incorporated to increase levels of gene transfer and expression. Although the exact mechanism of gene delivery is unclear and differs for each receptor, the pathway from the cell surface to the nucleus involves various endosomal compartments resulting in the transport of the DNA to the nucleus for expression [16] (**Figure 1**). There are many areas to consider when designing such molecules:

- size
- DNA condensation
- route of administration
- nuclease stability
- target sites
- *in vivo* deposition
- cell-binding
- internalisation
- intracellular trafficking

This review aims to address some of these areas. There are many patents in this area (see below), primarily based upon similar ligand-poly-L-lysine conjugates. The payoff for the most successful of these is potentially enormous and will probably result in cross-licensing and collaborative agreements.

2.1 DNA binding element

The way the DNA is attached and held in the complex is crucial to the stability of the vector (*in vitro*-in culture media, *in vivo*-in serum and within the endosomes-once inside the cell) and to the physical size of the complex. Polycationic chains are widely used to neutralise the negative charges of the DNA phosphodiester backbone and allow its condensation into a highly compact form. The more compact and small the complex is, the less chance there is that it will become engulfed by macrophages and be destroyed through the reticulo-endothelial system. The higher the positive:negative charge ratio, the more stable the DNA is to endonuclease degradation as the phosphodiester bonds are less exposed. Additionally, charge neutralisation of the DNA by polycations allows interactions with the negatively charged cell membrane. The polycation/DNA complex resembles a toroid or 'doughnut' structure (as seen by electron microscopy [17]) with a diameter ranging from 10 - 100 nm, which in some cases can be smaller than a virus. Commonly, Poly-L-lysine is chemically linked to the targeting ligand and this is relatively inexpensive to make. Studies have demonstrated the relationship between poly-L-lysine length and DNA stability or vector function (reviewed in [18]). Poly-L-lysine lengths of 8 residues or less have been shown to result in complexes of up to 3 μm in size, far too large to be taken up by RME [19].

The procedure of complexing the DNA to the polycation has also been studied in an effort to produce better molecules [18]. Generally, DNA and poly-L-lysine spontaneously associate to form a soluble complex. Poly-L-lysine has also been used to compact DNA into liposomes to make the smaller. Kabanov and Kabanov [20,101] have patented variations on polylysine with

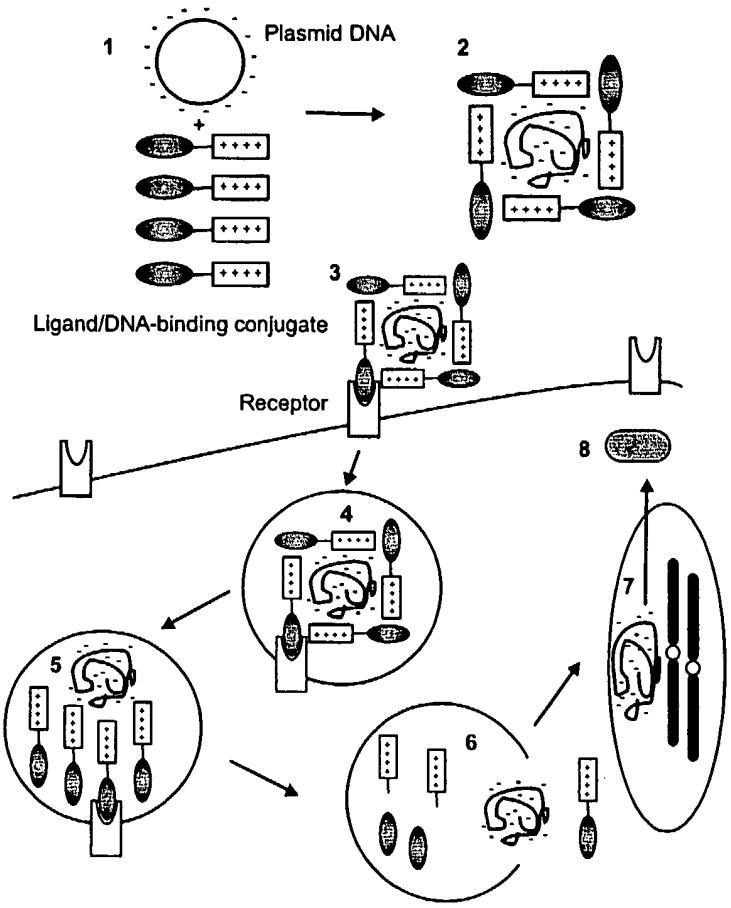


Figure 1: Schemetic diagram, taken as a summary from many sources, illustrating the general route of gene delivery by receptor-mediated endocytosis. The plasmid DNA, carrying the desired gene is complexed with the ligand-polycation (1) forming a gene transfer polyplex of size 10 - 100 nm (2). This binds to the specific receptor on the target cell (3) which is internalised and forms an endosome (4). Endosomes acidify and cause the break up of the complex (5). Endosomes release their contents into the cytosol, a process dramatically improved by the inclusion of osmolytic agents such as defective adenovirus (6). The DNA, some of which is still bound to the polycation, is localised to the cell's nucleus, a process aided by nuclear localisation sequences (7). Gene expression occurs (8), but the DNA is eventually lost as there is no active mechanism to retain it.

alternative polycation polymers for the use of DNA delivery. Other synthetic polymers such as polyamino chains with a glucose backbone [21] and polyethylemine have been developed. Cationic polysaccharides such as chitosan, which can bind DNA and have lectin specificity, are being studied as dual-function agents for transporting DNA and targeting cells [22]. Modifications such as these alter the kinetics of DNA uptake, but cannot be used in any recombinant approach. Further modifications such as polyethylene glycol (PEG) derivitisation have been patented in a bid to reduce any potential immunogenicity [102].

Commercially available poly-L-lysine preparations tend to be heterogeneous and not molecularly defined. Therefore, more recently, researchers have used naturally-occurring DNA binding proteins (see below). Histones and protamines are highly basic, small, compact proteins, with a high capacity for DNA, but are difficult to produce recombinantly. Histone H1 was found to be superior to the H2-H4 histones as a DNA carrier for liver gene delivery [23]. The sperm cell DNA compacting protein, protamine from humans, was used highly effectively in the form of an antibody-protamine fusion protein produced by mammalian cell culture [24]. The basic high mobility group protein (HMG-1) is being studied as an alternative carrier in recombinant approaches [25], with expression and activity demonstrated in *Pichia pastoris*. A basic peptide (sequence 'SPKRSPKRSPKR') derived from the histone H1 sequence [26] and a *de novo* designed sequence ('YKAK$_8$WK') based on the spermidine structure have been used [27]. Gottschalk *et al.* [27] found that this sequence was ineffective without the presence of an endosome disruption peptide (see below) and the fully functional vector was only 10-fold less effective than an adenovirus vector with 25 - 30% gene transfer levels in HepG2 cells. The yeast DNA-binding protein Gal 4 has been used quite ingeniously, despite its lower capacity for DNA (see below [28]). There are also examples of where DNA has been directly linked to the ligand in an approach termed 'antifection' (see below [29]).

DNA intercalating agents have been used as DNA carriers. Molecules such as acridine derivatives [30], ethidium bromide homodimers [31] and benzoquinone can complex to DNA, but there are concerns about the stability of the complex *in vivo* due to the DNA being non-condensed and susceptible to nuclease attack.

Since there are no packaging requirements, size limitation is not an issue with polyplexes. DNA constructs up to a size of 48 kbp have been reported to have been delivered *via* the transferrin receptor (see below) [32].

The nuclear membrane remains a barrier to gene delivery, as microinjection of DNA into the cytosol results in no expression compared to microinjection into the nucleus [26]. Cells which undergo mitosis after DNA exposure show a higher level of gene expression. Nuclear transport is accomplished by trafficking with a nuclear localisation signal (NLS). These take the form of a short stretch of lysines or arginines (e.g., KKKKPRK in the SV40 Large T antigen). These NLSs are transferable with proteins as large as 250 kDa being imported into the nucleus when 'tagged' with this peptide. The lysine-rich sequences used generally serve as satisfactory NLSs as well as DNA binding functions.

2.2 Endosome-disruptive functions

Gene expression levels and periods were initially found to be low by RME delivery of polyplexes and further research showed that the major rate limiting step was the escape of DNA from endosomes and transport to the nucleus (**Figure 1**). This was previously noted for the delivery of toxins in immunotoxins. It was found, then, that co-delivery of replication-defective adenovirus particles greatly increased the rate of endosomal escape. When applied to gene delivery, there was a 200-fold increase in gene expression levels and an increase in levels of transfection to 95% for the cells under study [33,103,104]. This was because the co-internalised adenovirus caused endosome disruption, releasing adenoviral protein (and DNA) into the cytosol. These endosome-disrupting functions are present within the adenovirus coat protein and occur in response to the pH decrease in the endosomes. When the adenovirus is linked to the gene transfer polyplex *via* an antibody bridge, the rate of gene transfer is improved a further two orders of magnitude [34], perhaps due to the use of a more effective adenoviral NLS. Other methods to link the adenovirus to the gene transfer complex include biotin-streptavidin bridges [35] and chemical linkers [36]. Alternative viruses, such as rhinovirus [37] or naturally-occurring proteins, have endosomal lysis/osomolytic functions, including influenza haemaglutinin (HA) or MS2 phage capsid proteins [105]. Peptides derived from the HA protein have been shown to cause endosomal lysis, but not as effectively as a whole adenovirus. Examples of these include 'GLFEAIAGFIENGWEGMIDGGGC' used in transferrin conjugates [38]. *De novo* designed peptides, based on the amphipathic nature of the haemaglutinin peptides have also been incorporated, such as 'GLFEALLELLESLWELILEA' [27]. Agents such as these have been described in conjunction with alternative DNA delivery vectors to form systems such as SPET-synthetic peptide enhanced delivery [39]

Many toxins have natural endosomal translocation domains which function distinctly to transport proteins across the membrane rather than to lyse the endosome. The 19 kDa diphtheria toxin translocation domain has been used specifically to augment DNA transfer, complexed with poly-L-lysine [40], whereas an existing antitumour *Pseudomonas* exotoxin immunotoxin was modified to deliver a DNA binding protein/plasmid complex, rather than the toxic catalytic domain (see below) [28]. The use of cholera toxin as a delivery and possible translocation domain has also been described as part of a patent for gene delivery to mucosal cells. The B-chains of the cholera toxin multimer may aid translocation across the cell membrane by forming a pore (see below [106]).

In many of the systems studied, the drug chloroquine was used to increase gene expression levels where endosomal processing was involved. Chloroquine is a weak base which neutralises acid compartments. It inhibits hydrolases found in lysosomes and inhibits the fusion of lysosomes with endosomes, thus reducing degradation of their contents and increasing DNA stability.

A different approach to promote release of endosome contents was patented by Berg *et al.* [107]. Here, a photosensitising compound is co-transfected with the DNA, followed by treatment of the cells with light at a certain wavelength. Light-activated, chemically-induced membrane disruption occurs, resulting in endosome release. This can be used for DNA or protein delivery, but may not find wide application *in vivo*.

2.3 Cell-specific ligand

One of the main attractions of this approach is the wide range of ligands/receptors which could be utilised for gene delivery. Examples of systems under study are presented in **Table 1**. This list is growing as fundamental advances in cell biology uncover new receptors and cell determinants. The various groups of ligands will be discussed in the context of the tissues targeted.

Table 1: List of ligands used in receptor-mediated polyplex vectors, the receptors targeted and the cell types expressing that receptor.

Ligand	Receptor	Cell type
Asialoglycoprotein (ASOR) asialofetuin Galactose/lactose	Asialoglycoprotein receptor (ASGP-R)	Hepatocytes (Parenchymal liver cells)
Lactoferrin	Lactoferrin receptor	Hepatocytes (Parenchymal liver cells)
Malaria circumzoite protein	Unknown	
α1-Anti-trypsin peptide	Serpin enzyme complex receptor	
Insulin	Insulin receptor	
Reconstituted sendai virus	Unknown	
Transferrin	Transferrin receptor	Malignant cells, glioma epithelial cells
Mannose	Mannose receptor	Macrophages
Lectins	Various	Lewis lung carcinoma
Epidermal growth factor (EGF)	Epidermal growth factor receptor (EGF-R)	Breast and pancreatic cancer
Folate	Folate receptor	Ovarian cancer
Steel factor	c-kit Receptor	Stem & haematopoietic cell
Peptides containing 'RGD'	Integrins	Epithelial cells
α2-Macroglobulin	α2-Macroglobulin receptor/low density lipoprotein receptor	Liver, intestinal, smooth muscle, neurone and fibroblasts
Cholera toxin subunit B	GM1 receptor	Mucosal epithelia
Adenovirus pentone base	Unknown	Epithelial cells
Anti-CD3/CD4/CD5/CD7 antibodies	CD3, CD4, CD5, CD7	Lymphomas
Anti-EGF-R antibody	EGF-R	Breast and pancreatic cancer
Anti-ErbB2 antibody	Erb B2/Her-2	Breast and pancreatic cancer
Anti-polymeric immunoglobin receptor antibody	Polymeric immunoglobin receptor (pIg-R)	Lung epithelial cells
Polyclonal anti-Fc-R antibodies	Fc-Receptor (Fc-R)	Alveolar macrophages (lung)
Ch1.7 antibody	190 kDa cell surface glycoprotein	Neuroblastoma cells
Anti-CD34 antibody	CD34	Haematopoietic stem cells
Anti-trombomodulin antibody	Trombomodulin	Lung endothelial cells
1E3 antibody	Tn Cryptantigen	Cancer, HIV infection, haematopoietic disease
Anti-idiotypic antibodies	Idiotypic antibodies	B-cell lymphomas

3. Systems under investigation

3.1 Gene delivery to the liver

One of the primary targets for gene delivery is the liver, which is the affected organ in diseases such as phenylketouria (PKU), haemophilia and hepatitis infection. The liver is the largest gland in the body, making up about 2% of the body weight. It is central to the metabolism of proteins and lipids, hence is an important commercial target for gene therapy. The ASGP-R is highly expressed on hepatocytes and has become a model receptor for the study of RME and internalisation [11]. The receptor interacts with glycoproteins that have terminal galactose residues. Wu and Wu synthesised a polyplex consisting of the desialated orosomucoid and poly-L-lysine and showed gene transfection of the transformed hepatocyte cell line HepG2 [13]. This was the first example of a successful gene delivered by this method. Reporter gene delivery experiments *in vivo* showed that 85% of the injected DNA was taken up by the liver by 10 min [41]. A great deal of research has followed, including *in vivo* gene delivery of albumin to rats with LDL receptor deficiency [42,108]. An average of 1000 copies of the plasmid were found per hepatocyte resulting in a level of 34 μg/ml human albumin in the serum of animals 2 - 4 weeks after injection and partial hepatectomy. Since then, many strategies based on this system have been patented, such as oligonucleotide delivery for antiviral therapy [109] and adenovirus enhancement [110]. Since one-fifth of the cardiac output flows through the liver per minute, this organ is amenable to *in vivo* gene delivery. Even in the absence of specific targeting, many molecules can be delivered to the liver, although not as efficiently internalised as those that are receptor-directed.

Most of the DNA delivered by this receptor was shown to be degraded, resulting in short-lived (4 days) and low levels of transgene expression. A partial hepatectomy stimulated longer expression, up to 11 weeks in some cases. This was found to be due to increased DNA stability and not due to replication or integration [16]. Further work by others have shown that the intracellular route taken by the DNA complex is not the same as that taken by the free ligand [43] resulting in unpredictable intracellular trafficking.

The human methylmalonyl-CoA mutase gene was delivered to rat liver cells by ASOR-poly-L-lysine [44]. Failure to correct the disease methylmalonic acidaemia is fatal. A staggering 95% of the injected dose accumulated in the liver, once again illustrating how this organ is amenable to this approach. After 6 - 24 h, the blood levels of enzyme increased 30 - 40% over background, although repeated doses were necessary to keep up potentially therapeutic levels.

Lactoferrin has also been used as targeting ligand, in combination with poly-L-lysine. This protein was seen to be better for liver targeting compared to transferrin [45] (whose uses are described later).

An interesting approach to delivering genes to the liver *via* the insulin receptor was by chemically derivatising albumin, making it positively charged and complexing it with the DNA and insulin [46]. This complex was able to transfect HepG2 cells, but it is difficult to compare this to other systems. Insulin was used to target the liver in a more elegant process patented by the Medical Research Council (UK) [111]. In one example of the invention, a transcription factor is fused to the hormone binding domain of the oestrogen receptor to generate a chimeric transcription factor. This is delivered to the target cell by an antibody. The polyplex is delivered to the same cells by a second antibody of different specificity. The DNA in this polyplex contains the gene (in this case a reporter gene) under the control of the transcription factor delivered by the first antibody. When both complexes are inside the target cell, gene expression will be active in the presence of oestrogen. This results in hormone-responsive gene delivery and expression. The use of two antibodies ensures that cell targeting is specific, as non-specific cells picking up one of the targeting agents will not have active gene expression. This idea is very attractive and adds another level of specificity, but is yet to be backed up with experimental evidence.

Gene transfer to the liver using galactosylated poly-L-lysine showed impressive gene delivery without the need for a partial hepatectomy [47,112]. The factor IX gene, driven by the phosphoenol pyruvate carboxy-kinase (PEPCK) promoter was introduced iv. into rats. The size of the complex was very small (10 - 12 nm) and resulted in the presence of the plasmid DNA episomally for up to 32 days, and the presence of the mRNA and protein for up to 140 days. Gene expression was induced by feeding rats with a high protein/carbohydrate-free diet. Similarly-sized complexes were made using triantennaryoligosaccharides linked to poly-L-lysine, resulting in high levels of gene expression. The high levels of gene expression and the impressive time periods for expression may be related to the size of the small

complexes used compared to the ASOR polyplexes, since the same receptor is targeted. Other carbohydrate-derived targeting complexes have been under study including lactose. Galactose-Histone complexes [23] were 11-times better at delivering reporter genes than ASOR ligands.

The α2-macroglobulin receptor/low-density lipoprotein receptor complex binds and endocytose a wide range of proteins, some of them as a complex with α2-macroglobulin. The α2-macroglobulin receptor is a large complex predominantly expressed on normal liver, smooth muscle cells, neurones and fibroblasts. This receptor was the subject of early studies and subsequent work has shown that the reporter gene, luciferase can be delivered and expressed in these cells by an α2-macroglobulin- poly-L-lysine complex [48]. The wide range of ligands taken up by this receptor make this an attractive target for the delivery of a 'cocktail' of genes, each complexed to a different ligand.

Another receptor complex targeted for gene delivery was the serine protease inhibitor (serpin) enzyme complex receptor (SECR) [49]. This receptor binds to conserved sequences on α1-antitrypsin and other serpins. A peptide based on this conserved sequence was used as a targeting ligand for poly-L-lysine conjugates which resulted in small (18 - 25 nm) complexes. Good levels of gene expression were achieved cells that express the receptor at high levels, such as the liver cells HepG2 and HuH7.

Ding *et al.* [50] reported that the malarial circumsporozoite protein (that covers the surface of the sporozoite form of the parasite), binds specifically to hepatocytes. Recombinant forms of this ligand have been chemically linked to poly-L-lysine to produce a gene delivery vector. Gene expression *via* this route is lower than the levels seen with the other methods, but is increased in the presence of adenovirus particles. Gene delivery to other cell-lines such as HeLa, NIH3T3 and K562 was also shown, suggesting a yet undiscovered receptor is being utilised.

Particles, which more closely resemble viruses, such as ligand-targeted liposomes, are being studied. These include asialofetuin-labelled liposomes [51], galactosylated lipopolyamines [52] and reconstituted sendai viruses [53]. These all have liver-cell specificity. Transfection is more efficient than with untargeted liposomes, but the inclusion of NLS sequences or fusogenic peptides does not having a potentiating effect [51].

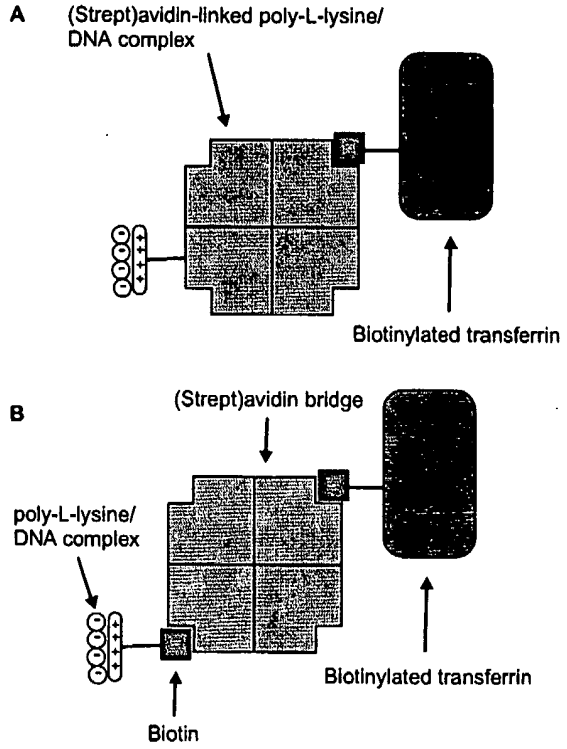
3.2 Gene delivery to tumour cells

The transferrin receptor is expressed on many endothelial cell types but is highly elevated on some tumours, including gliomas and haematopoietic tumours. Many of the early gene delivery experiments were carried out targeting the transferrin receptor, which has been a widely studied receptor for the delivery of many agents. Gene transfer to K562 haematopoietic leukaemic cells was achieved with a transferrin-polycation (poly-L-lysine or protamine) conjugate [54,104]. This work pioneered many new concepts, including the use of virally-derived fusogenic peptides as an alternative to whole adenoviral particles [33]. It was shown that the haemagluttinin-derived peptide from influenza virus was able to increase gene expression levels 10^2 - 10^4 times [38]. These 'fusogenic' peptides form membrane-disrupting helices under acid pH conditions, promoting endosomal lysis and gene delivery. However, only 10% transfection rates were seen, compared to the 90% seen with whole adenovirus. Also, these peptides seemed to be more toxic than adenovirus.

Delivery of genes *via* the transferrin receptor became known as 'transferrinfection'. Transferrinfection of melanoma cells with the gene for interleukin-2 resulted in a successful tumour vaccine which protected the animals from further tumour challenges [55,113]. A variation on the transferrin theme by Schoeman *et al.* [56] was to use the high affinity binding molecule streptavidin to cross-link the ligand-targeted cells with the condensed DNA complex (**Figure 2**). Biotinylated transferrin was used to target the cells, followed by the addition of biotinylated poly-L-lysine/DNA. The poly-L-lysine had 70 residues per chain and the transferrin had 1 - 2 biotins per molecule. Gene expression levels were significant, but the number of cells transfected was not described. This method was about 100-times more effective than avidin-poly-L-lysine combined with biotinylated transferrin. Other high affinity pairs of molecules have been suggested in a patent to link cell targeting ligands to DNA carrier. These include enzymes/peptide inhibitors and antibodies/antigens [114].

Neuroblsatoma cells have been found to express a 190 kDa cell surface glycoprotein, which is picked up by the monoclonal antibody chCE7. A poly-L-Lysine conjugate of this antibody was able to deliver luciferase genes at a transfection rate of 1 - 5%, about 2-fold lower than liposomes, but with 105-fold higher levels of expression [57]. To increase the levels of gene

Figure 2: Avidin or streptavidin has been used to link the DNA carrying element of gene transfer complexes to the targeting element. This can be done directly, where the ligand is biotinylated and cross-links to the DNA complex, or indirectly, where both components are biotinylated and are bridged by streptavidin. Steric constraints suggest that the latter may be more effective for gene delivery.



expression using the liposomes, toxic levels of cationic liposomes would have to be administered, whereas poly-L-lysine conjugates are non-toxic. The interferon-γ gene was tested as a more biologically relevant gene and it was found that HLA expression increased to higher levels than would have been achieved if 1000 IU/ml of pure exogenous IFN-γ was applied. Thus, targeted gene expression of IFN-γ proved much more effective and resulted in cytotoxic T-cell responses *in vitro*.

The normal liver expresses the epidermal growth factor receptor (EGF-R), but this receptor is highly elevated in many squamous cell carcinomas including breast and lung. The monoclonal antibody B4G7, which is internalised by EGF-R, was used successfully to deliver the CAT (chloramphenocol acetyl transferase) gene to tumour cells [58,115]. Further work showed that this system was able to deliver a suicide gene, herpes simplex virus thymidine kinase [59]. The transfected cells were 10-times more susceptible to the prodrug, gancyclovir, which resulted in 70% cell-killing, but these results are still a long way behind those achieved by viral delivery of prodrug-activating enzymes (VDEPT) (reviewed in [60]).

The natural ligand, EGF, has been used in a streptavidin-poly-L-lysine/biotinylated EGF system deliver the β-galactosidase gene aided by replication-defective adenovirus [61,116]. A four-fold improvement was seen in the presence of chemically linked replication defective adenovirus, with 14 - 99% cell transfection rates observed. Whether the proliferative effects of the EGF had any role in the transfection rates is not known, but this type of approach is promising for the delivery of p53 or k-*ras* gene to correct some lung cancers.

EGF-labelled liposomes have been used as an alternative to poly-L-lysine as the DNA carrying agent [62]. These targeted liposomes give only a 2-fold increase in the level of gene expression compared to non-targeted liposomes, *in vitro*, with high transfection rates (6 - 8%). However, how liposomes will behave *in vivo* needs to be addressed.

Fominaya and Wels suggested that, in general, effective gene delivery vectors would require about 10^5 - 10^6 adenoviral particles per target cell, or 10 - 100 μM fusogenic peptide, 50 - 100 μM chloroquine and a nuclear localisation sequence (usually sufficed by poly-L-lysine). An ingenious extension to their immunotoxin research [28,117] was to replace the catalytic domain of *Pseudomonas* exotoxin A immunotoxins with the DNA binding domain of the Gal 4 transcription factor (from yeast). Therefore, instead of delivering a toxin to the target cells, a gene could be delivered by the same route. The cell binding domain remains the recombinant single-chain Fv fragment against the erbB2 receptor, the immunotoxin's translocation domain can substitute for the adenovirus particles and the Gal 4 domain contains the DNA binding/NLS motifs (**Figure 3**). The Gal 4 fragment does not have as high a capacity for DNA as poly-L-lysine, as it binds through a sequence-specific zinc finger interaction rather than the non-specific electrostatic interactions of the latter. Any remaining negative charge was compensated by additional poly-L-lysine. Like the *Pseudomonas*-derived immunotoxins, these 'all-in-one' molecules express to high levels in *E. coli* and a high yield of recombinant product can be recovered. The added attraction of this system is that the amount and type of DNA which is bound in the complex can be controlled by the number of repeats of the Gal 4

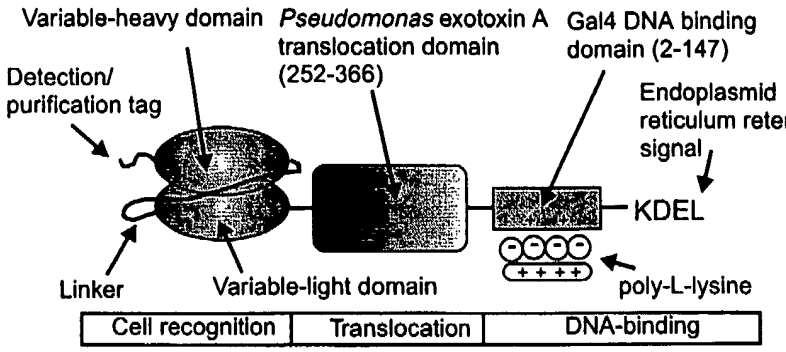


Figure 3: The modular assembly of recombinant gene transfer molecules was demonstrated by Fominaya and Wels [28,117]. Here, the cell recognition domain is a single-chain antibody, linked to a translocation from *Pseudomonas* exotoxin A and a DNA-binding domain from the Gal4 transcription factor. The 'KDEL' tetrapeptide sequence promotes retention of the complex in the endoplasmic reticulum, reducing the amount of protein lost to lysosomal degradation and targeting it to the compartment it translocated from, by retrograde trafficking.

recognition sequence. The more repeats, the more protein can bind to the same plasmid molecule. This may provide a way of controlling the kinetics of plasmid uptake. The Gal 4-DNA interaction is highly specific and of high affinity, possibly stabilising the DNA on route to the nucleus. Cos-1 cells were successfully transfected with this complex.

The ErbB2 (Her 2) receptor is restricted more to tumours than the EGF-R, and has been the subject of targeting with a humanised antibody (rhumAbHer2)-poly-L-lysine conjugate [63]. Gene delivery was almost 200-fold higher than with an irrelevant antibody. An NIH3T3 cell line transfected with the receptor as well as carcinomas were able to take up the gene specifically.

Glycoproteins of the mucin family, have been studied as targets. The Tn cryptantigen, which is expressed in cancers, haematopoietic disorders and on the HIV virus coat glycoprotein, is expressed in the model 'Jurkat' cell-line. This is due to a defect in the Tn-processing galactosyl-transferase. Gene delivery through this receptor has been accomplished using the 1E3 monoclonal antibody linked to poly-L-lysine [64]. Adenoviral particles increased transfection efficiency to 60%. Treatment of the cell with sialidase (which removes sialic acid and exposes more Tn antigen) increased gene delivery levels; competing GalNAc reduced gene expression. The transferrin receptor is also expressed on this cell-line and a direct comparison showed the Tn-antigen mediated system to be better. There is a 10-fold higher level of Tn antigen on Jurkat cells compared to transferrin, but gene delivery was 40-fold higher. However, the presence of multiple epitopes on a single Tn-protein may account for the better targeting.

Anti-idiotypic antibodies represent one of the few true tumour-specific antibodies, with successful examples of their targeting evident in cancer immunotherapy. A natural development to this was to use these receptors as targets for gene delivery to B-cell lymphomas [65]. Poly-L-lysine conjugates of anti-idiotypic antibodies show highly specific gene delivery.

Various lectins have been tested as possible ligands for RME endocytosis of DNA by tumour cells *via* the cell surface. Concavalin A was found to work well when biotinylated, and linked to the poly-L-lysine conjugated antibiotin antibody [66]. Receptors for these lectins are over-expressed on many cancers such as Lewis lung carcinomas.

Small molecules can also be taken up by cellular receptors by a process called 'pinocytosis'. Folate receptors are often over-expressed on ovarian tumour cells and folate-labelled liposomes carrying poly-L-lysine condensed DNA have been successful in delivering genes [67] and antisense oligonucleotides [68] to the tumour cells. These complexes are relatively small for liposomes, 74 nm in diameter. Antisense EGF-R oligonucleotides were able to inhibit EGF-receptor expression in these cells and cause a 90% reduction in cell growth, suggesting that significant amounts of oligonucleotide DNA can be delivered by this route.

3.3 Gene delivery to lymphocytes

The CD3 receptor is expressed on 95% of T-cells at a level of about 10 - 40,000 molecules per cell. Its mitogenic binding results in a rapid rate of endocytosis: 420,000 molecules over a period of 24 h. Antibodies (OKT3, WT32 and UCHT-1) against the CD3 molecule, conjugated to poly-L-lysine were used to deliver a CMV-driven luciferase gene to T-cells [39] in the SPET/AVET system. Up 50% transfection rates were seen in Jurkat cells, increasing in the presence of

chloroquine and membrane-active peptides (10- to 100-fold). *In vivo*, 1000 - 2000 units of interleukin-2 were expressed from transfected Jurkat cells, which peaked at 24 h. Peripheral blood lymphocytes were transfected at a lower rate (5%).

A study compared the effectiveness of gene delivery to lymphoid cells *via* the CD3, CD34 and surface immunoglobulin receptors, using monoclonal antibodies in a technique called 'antifection' [29]. Although the transferrin receptor is prevalent on lymphoid cells, delivery by this route compared to these other receptors is about 1000-fold less effective. In this system, there is no DNA condensing agent as the plasmid molecule is directly coupled to the antibody. *In vitro* transfection results were not as good as conventional approaches, with 0.1% transfection rates at best. However, *in vivo* on spleenocytes, impressive 1 - 7% transfection rates were seen as detected by β-galactosidase expression and neomycin resistance. This compared well to the poly-L-lysine mediated system above [39]. There was no discussion about the size of these complexes or the stability of the DNA. A high affinity antiCD5 antibody (T101) has also been used, linked to poly-L-lysine to deliver a reporter gene to lymphocytes [69].

Steel factor is peptide ligand, which binds to the c-*kit* receptor on primitive haematopoietic stem cells. Streptavidin-conjugated poly-L-lysine/DNA complex was targeted to cells by biotinylated steel factor [70]. After 2 h incubation, the maximal transfection efficiency approached 90% with maximal gene expression after 30 h. The gene expression was improved almost 10-fold by the addition of adenovirus to promote DNA endosomal escape. In this example, the strategy allowed the mixing of any biotinylated ligand to the DNA complex to deliver genes to a wider range of cells. Transferrin was used to illustrate this. The high efficiency of gene delivery could be used for the purging of bone marrow *ex vivo* by the delivery of suicide genes.

Gene delivery directly through the CD3 receptor has been shown to result in low levels of expression attributed to the induction of TNFα-mediated apoptosis, caused by binding the CD3 receptor [71]. It was shown that this effect could be counter-acted by the inclusion of anti-TNFα-antibodies during transfection, resulting in increased proliferation rates of transfected lymphocytes.

Bispecific antibodies (bsAbs) present an alternative way to deliver genes to lymphocytes. This is exemplified by the use of an antiCD3/anti-FLAG bsAb to target FLAG-peptide bearing adenovirus to CD3-expressing cells [72]. However, this is still re-direction of infectious virus.

In a study to find alternative ways to deliver toxins without the problems of toxin immunogenicity, Chen *et al.* [24] extended the ligand-cation polyplex research area into the area of recombinant fusion protein construction. They created a recombinant antibody (Fab) against the HIV coat protein gp120, fused to the human DNA binding protein protamine. Recombinant fusions have the advantages of being a homogeneous species of purified molecule, which can be rationally designed using the tools of protein engineering. This completely human-derived fusion protein polyplex was able to deliver the gene for a toxin, *Pseudomonas* exotoxin A, resulting in cell-targeted cytotoxicity. Highly cationic polypeptides are notoriously difficult to produce recombinantly, making recombinant production of such gene therapy vectors troublesome, although the advantages are attractive.

Lymphocytes can also be targeted by interleukin-2, as has been seen in tumour targeting. Gene delivery fusion proteins (GDFPs) based on IL-2 and Gal4 (as the DNA binding domain) have been patented and shown to localise plasmid DNA to cells, but no expression was reported [118]

3.4 Gene delivery to macrophages

Alveolar macrophages play a role in lung homeostasis and pathogenesis of disease. Cognate polyclonal antibodies have been produced against the Fc-receptor of these cells and used chemically conjugated to a 30 kDa poly-L-lysine chain [73]. Gene expression in purified alveolar macrophages of β-galactosidase was 5-times that of the background and increased further in the presence of chloroquine. Gene delivery was specific as it was competed by unconjugated antibody and did not transfect Fc-R-negative cells.

Peripheral blood macrophages possess mannose receptors which have also been targeted for gene delivery [74]. Mannosylated poly-L-lysine was the delivery vector and expression was short-lived, peaking at 4 days and dying out 16 days. Expression was confirmed to be confined to macrophages as the localisation of non-specific esterase enzyme marker correlated with gene expression. The DNA complexed was well-neutralised with about a 1:0.9 ratio of DNA:poly-L-lysine. The chain length was about 100

Table 2: Some examples of promoters that could be used in conjunction with targeted gene delivery, and the diseases which are suitable.

Disease/tissue	Gene	Promoter
Insulin-dependent diabetes mellitus/pancreas β-cells	Insulin	Insulin promoter
Hepatoma/liver	Cytotoxic	α-Fetoprotein
Metabolic diseases/liver	Factor VIII, X, PKU	Albumin
Muscular dystrophy/muscle	Dystrophin	α-Actin
Lymphomas/B-cells	Cytotoxic	Immunoglobulin heavy-chain
Cancer/breast	Cytotoxic	Erb B2
Melanoma/melanocytes	Cytotoxic	Tyrosinase
Lymphomas/T-cells	Cytotoxic	T-cell receptor
SCID/T-cells	Adenosine deaminase	T-cell receptor
Colorectal cancer/GI tract	Cytotoxic	Carcinoembryonic antigen

residues with about 1% being glycosylated. Electron microscopy showed the whole complex to be a favourably small 10 - 20 nm in diameter. Such vectors could be used to treat reticulo-endothelial storage diseases such as Gaucher's disease.

Antisense oligonucleotides have also been delivered by this route [75], with the ablation of TNF-specific mRNA. This adds support to the view that this method may be able to achieve significant levels of delivered oligonucleotide.

3.5 Gene delivery to epithelial cells

Respiratory epithelial cells express the polymeric immunoglobulin receptor (secretory component) which is involved in transporting antibodies across mucosal layers. An antibody (Fab)-poly-L-lysine complex was used to deliver genes to epithelial cells *in vitro* [76] and *in vivo* [77]. Injection of the complex into rats resulted in high levels of gene expression in the surface epithelium and submucosal glands of the lung and with some expression in the liver, demonstrating excellent targeting. The level of expression lowered upon multiple injections due to the illicitation of antirabbit antibodies [78]. The important findings were that there were no anticomplex or anti-DNA antibodies raised in response to the treatment. There was no activation of complement, although it is thought that free poly-L-lysine can activate complement by the alternative pathway. Airway epithelial cells represent a good target for gene delivery as transfection efficiencies in the region of 10% may be enough to relieve the symptoms cause by cystic fibrosis disease [79].

The gastrointestinal (GI) epithelium is an important target in terms of absorption/secretion and digestive disorders and malignancies. The transferrin receptor can be used to deliver genes to GI epithelial cells and colon carcinoma cells [80]. High levels of expression of the serum protein α_1-anti-trypsin were achieved, opening up the possibilities of correcting acquired serum protein deficiencies by this route as well as the liver.

Lectins can also be used to target epithelial cells as well as tumour cells. Concavalin A linked to histone proteins have shown the most promising results by this route [81]. The natural affinity of adenovirus for epithelial cells has been exploited in the use of the virus fibre and pentone coat proteins as targeting/fusogenic proteins [82].

Epithelial cells are specifically bound by the invasin protein produced by some pathogenic bacteria as part of their infection process. Invasin has been used as a targeting ligand in conjunction with the Gal4 DNA binding protein to achieve specific reporter gene expression [83]. In another example of pathogen-derived targeted vehicles, the cholera toxin, which is

composed of a AB_5 hexamer, has been used to deliver DNA to the gastric epithelial cells normally attacked by the toxin itself. The cell binding domain (B subunit) which targets the GM1 glycolipid receptor on mucosal epithelia, was linked to poly-L-lysine as used to deliver functional cystic fibrosis gene mRNA, reducing cystic fibrosis symptoms [106].

3.6 Gene delivery to other cell types

A variety of ligands were screened for most effective binding to myogenic cells. Transferrin was selected and used to deliver genes to these cells opening up the possibility of targeting a correction for Duchenne muscular dystrophy [84].

Tissue-specific lung gene delivery to lung endothelial cells has been achieved using a monoclonal antibody-poly-L-lysine conjugate. The antibody recognised the cell surface thrombomodulin [85]

Integrin research is expanding rapidly as they are found to be involved in a great many cell-cell interactions. These are particularly embryogenesis, tumour metastasis, wound healing and T-cell function. Cyclic peptides containing an integrin recognition sequence 'Arginine-Glycine-Aspartate' (RGD) has been used, fused to a poly-L-lysine chain [86,87,117]. The entire targeting construct was chemically synthesised, making this a simple molecule to make and develop. High levels of gene expression were seen in a variety of cell lines bearing the receptor.

A general cell targeting construct was created by fusing the immunoglobulin-binding domain of protein A to the coat protein of sindbis virus [88]. Cell targeting is achieved by incubating the chimeric virus with a cell-specific monoclonal antibody. This allows cell specificity to be altered easily by changing the antibody.

4. Conclusions

Presently, this approach to gene delivery is much less efficient than viral gene delivery. However, under optimal conditions, enough gene product may be produced to give a therapeutic benefit (e.g., suppress a phenotype or destroy a tumour). With current technologies, it is very likely that multiple doses will be needed to maintain adequate expression levels. If the complexes are not immunogenic (i.e., human proteins used), this may be a viable option, perhaps more desirable in that a controllable, safer treatment modality is achieved. Therefore, the potential drawbacks are compensated by the significantly lower risk levels associated with this method.

It is difficult to compare the efficiency of different gene delivery systems, especially between those that target different receptors as each delivery route is different. Different reporter genes are used and groups using the same gene, e.g., luciferase, describe different ways of presenting the results. Nevertheless, one can get an idea of how good a system is by measuring the time of gene expression, the percentage of cells transfected and, ultimately, the curative effects in an animal model *in vivo*. Although better targeted, by these measurements, they lag behind viral methods.

However, the practically unlimited size restraints on the size of DNA deliverable by these receptor-mediated polyplexes can give these systems a major advantage over viral targeting. Genes for large proteins, sets of genes or genes with complex regulatory sequences could easily be accommodated. The expression of integrated genes tends to be higher for those that contain the correct intron structure to allow proper processing. This is true for transgenic animal gene expression [89].

This is certainly a growth area in gene therapy. Many of the targeting constructs are similar in basic structure (ligand-polylysine/polycation-adenovirus/fusogenic peptide), which has been demonstrated to be very effective. Many of the patents in this area describe the novel invention (e.g., a specific ligand or coupling method), followed by a description of general applicability. Therefore there seems to be a large degree of overlap between patents.

Research into tissue-specific targeting of tissues such as the liver, bone marrow stem cells and macrophages is well advanced, with good prospects for clinical testing. Tumour targeting of genes is also progressing well, basically following the same lines of receptor targets as previous immunotoxin research. Genetic delivery of toxins to tumours may prove to be more effective than immunotoxins.

5. Expert opinion

Gene delivery by ligand targeted receptor-mediated endocytosis of polyplexes should find its way into some main line gene therapy treatment schemes by virtue of its superior specificity, lower risk and reduced size limitations. However, in order to achieve the levels of gene transfection and expression seen

with retroviral vectors, further advances need to be made in fields such as mammalian artificial chromosomes [90]. Potentially, once genes are specifically delivered, they may be maintained for long periods of time in a way analogous to bacterial plasmids or artificial chromosomes (BACs) or yeast artificial chromosomes (YACs)

The powerful combination of cell-specific targeting *via* receptors and promoter specificity may allow an even higher degree of specificity resulting in 'super--specific targeting' of genes to cells with very little non-targeted expression. For example, the targeting of genes to the liver *via* the asialoglycoprotein can be combined with the use of the liver-associated albumin promoter, or the genes delivered to tumour cells through the erbB2 receptor may be placed under the transcriptional control of the erbB2 promoter. There is already evidence of this approach being used [42], but there are many more possible tissue or tumour specific promoters which could be utilised in this way (**Table 2**).

Library selection techniques such as phage display will ultimately yield new tissue or tumour-specific antigens to expand the options available for cell targeting. Organ-specific peptides have been isolated using elegant cell-selection strategies [91] or *in vivo* panning [92]. The more ligands we have at our disposal, the better options we will have for specific targeting. Combinations of specificities may overcome antigen heterogeneity problems.

Finally, advances in heterologous gene expression systems will also expand the options available in the construction of such delivery vectors. The ability to produce highly basic proteins, which are notoriously difficult to express recombinantly at high levels, combined with protein engineering and rational design will provide the researcher with more advanced tools for constructing effective gene delivery agents, such as those which are activated to bind tumours when processed by tumour-derived matrix metalloproteinases (as seen for recent retrovirus constructs) [93].

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Molecular therapy in pancreatic adenocarcinoma

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Pancreatic cancer is one of the commonest causes of death from cancer. Despite therapy with surgery, conventional chemotherapy, and radiation, 5-year survival for patients with this diagnosis remains poor. However, advances in the molecular understanding of this malignant disease over the past 5 years might lead to new treatment strategies. Strategies of gene therapy, antiangiogenic treatments, immunotherapy, and signal-transduction inhibition are in preclinical development. This review presents an overview of molecular therapy in pancreatic cancer.

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Pancreatic cancer (figure 1) is the fourth commonest cause of death from cancer in men and women, with 5-year survival for all stages of disease of less than 5%. Most patients with cancer of the pancreas are diagnosed at an advanced stage and are therefore not candidates for surgical resection.[1]

A pivotal randomised trial has shown that gemcitabine is more effective than fluorouracil in the alleviation of disease-related symptoms such as pain, low performance status, and weight loss.[2] A large phase III trial of gemcitabine alone or in combination with fluorouracil in advanced pancreatic cancer showed that combination treatment did not significantly improve median survival (6·7 months) compared with single-agent gemcitabine (5·4 months).[3] Therefore, single-agent gemcitabine continues to be the standard therapy in the palliative treatment of pancreatic cancer.

Several other combination chemotherapy regimens have been investigated in phase II clinical trials and have included: gemcitabine plus cisplatin (median survival 7·4–8·3 months); gemcitabine plus irinotecan (median survival 6·0 months); gemcitabine plus docetaxel (median survival not yet reported); and gemcitabine plus oxaliplatin (median survival 9·2 months). At present, a randomised phase II study to compare single-agent gemcitabine with combinations of gemcitabine with cisplatin, docetaxel, gemcitabine, or irinotecan is being done by the Cancer and Leukemia Group B.[4]

Combination treatment with radiotherapy and chemotherapy has been assessed in the treatment of locally advanced pancreatic cancer. Several cooperative-group trials have shown that chemoradiotherapy slightly improves median survival. Larger phase II randomised trials are needed to define the precise role and benefits of combination therapy in patients with locally advanced disease.[5]

Figure 1. Histological section of pancreatic adenocarcinoma.

Despite several phase II trials of chemoradiotherapy regimens, no clinically meaningful gains have yet been made in the treatment of pancreatic cancer. There is an urgent need for more effective therapy for patients with advanced disease. Potential approaches might include gene therapy, antiangiogenic agents, immunotherapy, and inhibitors of cell signalling. This review addresses advances in the molecular therapy of pancreatic cancer over the past 5 years.

Gene therapy

This technique aims to eradicate cancer cells by the manipulation of intracellular genetic material. Strategies might include attempts to restore the function of inhibited tumour-suppressor genes or inhibit the function of activated oncogenes. The theoretical basis for gene therapy is the assumption that elimination, or restoration, of the activity of a single gene product will reverse the malignant phenotype. However, this hypothesis ignores the evidence that pancreatic cancer results from the mutation of not one, but many genes. The effectiveness of gene therapy depends on the technical ability to inhibit or restore gene products in most of the tumour cells.

The genetics of pancreatic cancer make it one of the most complex malignant diseases, with more mutations than any other common tumour type. Some pancreatic

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Table 1. Common genetic mutations[1,8]

Gene	Frequency of mutation (%)
Oncogenes	
KRAS2	95
LSM1	87
AKT2	10–20
MYB	10
Tumour-suppressor genes or maintenance genes	
ARPC5	>90
P53	50–75
SMAD4	55
BRCA2	7–10
STK11	5
MAP2K4	4
TGFBR1	1
TGFBR2 (*MSI1* negative)	1
TGFBR2 (*MSI1* positive)	3
MLH1	3

tumours might have mutations in five or more genes. Mutations can occur in oncogenes, tumour-suppressor genes, or maintenance genes[6] and can subsequently activate oncogenes or inactivate tumour-suppressor genes, leading to a malignant phenotype (table 1).

Antisense strategies

Antisense gene therapy aims to prevent the transcription or translation of cancer-associated genes. It involves the production of short segments of deoxynucleotides that bind to target DNA or RNA to inhibit production of proteins.[9]

HRAS is the most commonly mutated oncogene in pancreatic cancer and many other cancers, and has thus been a therapeutic target for many studies of antisense sequences. Studies done in vitro have confirmed that transfection with plasmids that contain *KRAS2* antisense inhibits the growth of pancreatic-cancer cell lines.[10] In a phase II trial of the antisense inhibitor of *HRAS*, ISIS 2503, in 30 patients with advanced pancreatic carcinoma, the main toxic effects were mild thrombocytopenia and asthenia. Two patients had disease stabilisation, and median time to progression was 2 months (range 1–7).[11] In a follow-up trial on 48 patients, the toxic effects with the combination of ISIS 2503 and gemcitabine were similar to those seen with gemcitabine alone. Median survival was 6·6 months, and 14·6% of patients responded to combination treatment (with one complete response and six partial responses). Larger trials of this antisense agent are planned.[12]

LSM1 is an oncogene that is overexpressed in 87% of pancreatic cancers. An adenovirus engineered to express antisense RNA to the *LSM1* gene has been studied in vitro and in vivo, showing decreased expression of LSM1 mRNA and decreased anchorage-independent growth of pancreatic-cancer cells. A single intratumoral injection of the adenovirus significantly extended the survival of mice with severe combined immunodeficiency and pancreatic cancer.[7]

Replacement of tumour-suppressor genes

P53 is mutated in most pancreatic carcinomas, and has thus been the focus of several preclinical studies. Wildtype (unmutated) *P53* transduced into pancreatic-cancer cell lines by use of adenovirus vectors and retrovirus vectors causes growth inhibition and apoptosis.[13,14]

The proapoptotic gene *P73* is a member of the *P53*-gene family and, when overexpressed, binds to target sites in *P53* DNA, activates P53-responsive genes, and induces cell-cycle arrest and apoptosis. Rodlicker and Putzer[15] have shown that an adenoviral vector that encodes *P73* promotes apoptosis in several pancreatic-cancer cell lines, including cell lines known to be resistant to *P53* replacement.[15]

SMAD4 is a tumour-suppressor gene that is associated with a poor prognosis when inactivated in pancreatic cancer. Transfer of this gene by use of adenovirus to pancreatic-cancer cell lines deficient in *SMAD4* was associated with restoration of *SMAD4* expression and function, and inhibition of tumour growth was seen in mice transfected with the gene.[16]

Suicide-gene therapy

Also called gene-directed enzyme prodrug therapy, this strategy is a two-step process. First, a vector delivers a gene into the tumour cell that leads to expression of an enzyme. Second, a prodrug is administered that is activated selectively by the enzyme. Because the activating enzyme is present only in tumour cells, these cells selectively accumulate high concentrations of active, toxic drug. The most well-known example of this approach is the herpes simplex virus thymidine kinase/ganciclovir system.

Suicide-gene therapy has produced variable results in animal studies on pancreatic cancer. In human pancreatic-cancer cell lines, Wang and co-workers[17] showed that suicide-gene treatment substantially decreased survival of tumour cells. Makinen and colleagues[18] found similar positive results in vivo, as well as in vitro. However, other studies have not confirmed the efficacy of suicide genes in pancreatic-cancer cell lines.[19,20] Although this approach has not been assessed in clinical trials for patients with pancreatic cancer, results for other tumour sites have not been encouraging.[21,22]

Oncolytic-virus therapy

Replication-selective viruses are able to replicate preferentially in, and therefore lyse, cancer cells while sparing healthy tissue.[23] Adenovirus ONYX-015 preferentially replicates in, and kills, cells that have defective or deficient *P53* function (ie, 50–75% of pancreatic cancers). In a phase I/II clinical trial to assess intratumoral injection of ONYX-015 by use of endoscopic ultrasonography in combination with intravenous gemcitabine, 21 patients with advanced pancreatic cancer reported few procedure-related combinations. Two patients experienced partial responses, two had minor responses, six had stable disease, and eleven had progressive disease. These results indicate that oncolytic viruses could be a practical method of gene therapy in the future.[24]

Targeting apoptotic pathways

Apoptosis is essential for carcinogenesis and tumour progression. Two signalling routes can lead to apoptosis: the intrinsic pathway (initiated in mitochondria) and the extrinsic pathway (initiated by the binding of death-receptor ligands to specific death receptors on the cell surface).[25] Most approaches to gene therapy target the extrinsic pathway.

Katz and colleagues[26] studied the efficacy in vitro and in vivo of an adenovirus vector that targets TRAILR1 and TRAILR2, two members of the death-receptor subfamily thus initiating apoptosis in pancreatic-cancer cell lines and mice xenografts. The vector expresses a gene that encodes the death-receptor ligand, TRAIL, which binds to the death receptors and initiates apoptosis. Use of TRAIL in targeted therapies has been associated with toxic effects in healthy tissues, especially in the brain and liver. To promote the selective expression of TRAIL in cancer cells only, Katz and colleagues used a human telomerase reverse-transcriptase promoter. Telomerase is active in more than 85% of cancer cells, but not in healthy cells. Thus, under the control of a human telomerase reverse-transcriptase promoter, the TRAIL gene should be activated only in cancer cells. This method has yet to be tested in humans, but results in mice with pancreatic cancer have been encouraging.[26]

Immunomodulatory gene therapy

Cytokines can inhibit the development and progression of tumours, and systemic administration of cytokines can elicit antitumour effects but might also cause unacceptable toxic effects. Direct intratumoral injection of vectors that encode genes for cytokines might help avoid the systemic toxic effects associated with intravenous administration of immunomodulatory agents.

Several efforts have been made to achieve therapeutic concentrations of cytokines by intratumoral injection. Peplinski and colleagues[27] gave a recombinant vaccinia virus that encoded human interleukin 1β to mice with established pancreatic tumours. Both intratumoral and intravenous routes of administration of the virus caused a significant decrease in tumour size ($p<0·001$).[27]

A vector that encoded both interleukin 12 and a costimulatory molecule, B7.1, was associated with complete regression of tumour in 80% of mice with pancreatic-tumour xenografts. Moreover, when the mice were rechallenged with the same tumour cells, 70% of those previously cured remained tumour-free. This finding suggests that protective immunity had been conferred.[28]

Other investigators have tested the strategy of inducing pancreatic tumours to express the receptor for a cytokine, and then administering systemic or local therapy with that cytokine. For example, Kawakami and colleagues[29] injected pancreatic-cancer xenografts with a plasmid that encoded the α2 chain of the interleukin-13 receptor, followed by treatment with interleukin 13. The modified tumours became highly sensitive to the antitumour effects of interleukin 13.[29]

Table 2. Clinical trials of gene therapy

Trial	Developmental phase
CEA peptide 1-6D either emulsified in montanide ISA-51, or dissolved in granulocyte-monocyte colony-stimulating factor in HLA-A2-positive patients with adenocarcinomas from the gastrointestinal tract that produce CEA	II
SS1 (dsFv)-PE38 immunotoxin in patients with advanced mesothelin-expressing malignant disease	I
Autologous dendritic cells infected with recombinant fowlpox-CEA-TRICOM vaccine in patients with advanced or metastatic malignant diseases that express CEA	I
LMB-Immunotoxin in patients with advanced adenocarcinoma that overexpress Lewis-Y antigen	I

CEA, carcinoembryonic antigen.

Summary of gene therapy

Advances in the knowledge of the genetics of pancreatic cancer provide exciting new opportunities for the application of gene therapy. cDNA microarray analysis provides an opportunity to identify new gene targets. One study,[30] for example, has identified the overexpression of 103 genes that were not previously reported to be associated with pancreatic cancer.

However, more practically, the ability to construct vectors that are capable of safe, efficient, and selective gene transfer remains limited.[31,32] The intricate genetics of pancreatic carcinoma also complicate the present situation: pancreatic cancer is the result of cumulative and complex genetic mutations and restoration or deletion of single-gene function is unlikely to have a real clinical benefit, especially given that patients generally present with advanced disease. Table 2 lists current clinical trials of gene therapy for patients with pancreatic cancer.

Antiangiogenic strategies

Angiogenesis is a crucial step in tumour growth and metastasis and has therefore been investigated intensively, with inhibition of angiogenesis emerging as a valuable therapeutic strategy.[33]

Angiogenesis occurs when there is an imbalance of proangiogenic and antiangiogenic factors. Vascular endothelial growth factor (VEGF) is a proangiogenic glycoprotein that has a mitogenic effect on vascular endothelial cells and thus promotes tumour angiogenesis. Many trials have shown an association between tumour concentrations of VEGF and disease progression. Several agents that act at the level of VEGF and its receptors have been studied preclinically and in clinical trials.[34]

DC101, an antibody against mouse VEGF receptors, has been investigated in nude mice with human pancreatic cancer. Blockade of VEGF receptors by DC101 was associated with a significant decrease in the growth of primary tumours compared with untreated mice ($p<0·01$). After resection of the orthotopically placed tumour, analysis

showed decreased microvessel density (suggestive of an antiangiogenic effect) and increased areas of hypoxia. Furthermore, these effects were increased by the addition of gemcitabine to DC101.[35]

One of the most studied monoclonal antibodies against the VEGF receptor in humans is bevacizumab. In a large randomised trial on patients with colorectal cancer, this agent has shown a significant benefit for median survival when used in combination with chemotherapy (compared with chemotherapy alone, p=0·00003).[36] Bevacizumab has also shown a survival benefit in metastatic RCC.[37] Reported toxic effects included hypertension, proteinuria, and increased frequency of thromboembolism.

In a phase II trial, 40 patients with stage IV pancreatic carcinoma were given combination treatment with bevacizumab and gemcitabine; 33 were evaluable for response. The interim results showed that six-month survival was 74%; projected 1-year survival is 54%. Eight patients (24%) had partial responses with a median duration of 9 months, and 13 patients (39%) had stable disease. If these results can be confirmed in larger phase III trials, this treatment could vastly improve the outlook for patients with stage IV pancreatic cancer. Such trials are in development.[38]

Thalidomide also has antiangiogenic properties and has been assessed together with chemotherapy in patients with pancreatic cancer. The exact angiogenic pathways that thalidomide targets remain unknown, but substantial reduction of VEGF-stimulated growth of blood vessels has been observed in studies on animals. Thalidomide probably exerts its effects through anti-inflammatory pathways.[39] In a small phase I trial of patients with advanced pancreatic cancer whose disease had progressed while they were receiving gemcitabine, patients were given the combination of thalidomide and docetaxel. Median survival was 15 weeks, but patients with cancer refractory to treatment with gemcitabine might be expected to have poor outcomes. Phase II trials on thalidomide for advanced pancreatic cancer are planned.[40]

Preclinical studies continue to assess agents with antiangiogenic activity mediated through pathways other than VEGF. Multisynthetase complex auxiliary component P43 (also called endothelial-monocyte-activating protein II, EMAPII) is a tumour-derived cytokine that has potent effects on endothelial cells, including upregulation of tissue factor and modulation of tumour necrosis factor.[41] In vivo, EMAPII has antitumour activity in mice xenografted with human pancreatic-tumour xenografts. Treatment with EMAPII decreased intratumoral microvessel counts, suggesting that it inhibits angiogenic pathways (although the specific pathways are not yet identified).[42]

Several other agents with antiangiogenic mechanisms of action are under development in clinical trials. These include the small-molecule, tyrosine-kinase inhibitor of VEGF receptors SU112,[43,43] the aspergillus derivative TNP-470,[44] and the integrin inhibitor cilengitide.[45,46] These agents have not yet been studied in phase II trials on patients with pancreatic cancer.

Matrix metalloproteinases are a group of proteolytic enzymes that are important in the degradation of extracellular matrix—a key step in the process of angiogenesis. Therefore, inhibitors of these enzymes could be potential antiangiogenic agents.[47] Two large clinical trials of matrix-metalloproteinase inhibitors have been completed for patients with pancreatic cancer. However, neither trial found that they have significant clinical activity.[48,49] Preclinical studies with new matrix-metalloproteinase inhibitors, which might have higher activity, are under way.[50]

Summary of antiangiogenic therapy

VEGF has emerged as an important potential therapeutic target in pancreatic (as well as other) malignant diseases. Bevacizumab, an antibody that targets VEGF, has shown encouraging results in phase II clinical results. Large randomised trials with this agent in pancreatic cancer are planned. The potential benefits of bevacizumab in an adjuvant setting in pancreatic carcinoma have yet to be investigated, but an advantage is that the angiogenic process would be targeted earlier when cells might be more sensitive to the action of VEGF inhibitors.

Immunotherapy

Stimulation of a patient's own immune system to achieve an antitumour response can involve non-specific immune stimulation such as that seen in earlier studies. For example, Coley[51] used this method to treat patients with sarcomas who had severe bacterial infections.[51] In later studies, bacillus Calmette-Guerin was used in immunotherapy of patients with various malignant diseases.[52]

Immunotherapy can be either humoral (ie, involving antibodies) or cell mediated (ie, involving cytotoxic T cells). Immunotherapeutic agents that stimulate humoral immunity include monoclonal antibodies that target tumour-specific antigens.[53] Cetuximab, a monclonal antibody against epidermal-growth-factor receptor (EGFR), and trastuzumab, a monoclonal antibody against HER2, could be potential therapeutic agents for pancreatic cancer.

Cell-mediated immunotherapy requires the selection of a tumour antigen (or antigens) to produce a vaccine. At first, vaccines that used whole-cell lysates (and therefore several unspecified tumour antigens) were used. More recently, sophisticated technology has enabled the selection of specific protein targets and the development of agents specifically aimed at these targets. The selection of the tumour-associated antigen to exploit in making a vaccine is crucial:

Table 3. Tumour-associated antigens[44]

Antigen	Type	Frequency (%)
CA 19-9	Carbohydrate antigen	90
CA 242	Carbohydrate antigen	90
Mesothelin	Glycoprotein	89
LSM1	Oncoprotein	87
CEA	Oncofetal protein	85–90
GA733-1	Glycoprotein	85
HER2	Oncoprotein	27–80
KRAS2	Oncoprotein	75–100
MUC1	Glycoprotein	90
P53	Tumour-suppressor protein	40–70

an antigen should be selectively expressed by tumour tissue and not healthy tissue, and should have high immunogenicity (table 3).

Peptide antigens that could act as immune epitopes in pancreatic cancer include MUC1 (mucin 1) and CEA (carcinoembryonic antigen). MUC1 is a large glycosylated protein that is expressed on glandular epithelium. Preclinical and animal studies suggest vaccines that exploit this epitope can generate an immune response.[52] A clinical trial that enrolled patients with several tumour types, including pancreatic cancer, attempted to strengthen the immune response to MUC1 by transfection of MUC1 cDNA into dendritic cells, which are potent antigen-presenting cells. Immunisation with repeated injections of the autologous dendritic cells engineered to express MUC1 increased the frequency of mucin-specific, interferon-secreting CD8-positive T cells in some patients. These findings suggest an immune response in these patients, and further studies of this technology are under way.[54]

The CEA glycoprotein is expressed in small amounts in healthy epithelium of the colon and is overexpressed in several malignant diseases. Preclinical and clinical studies of vaccines that use the CEA epitope have been completed. Most clinical trials have involved patients with colorectal cancer and several have shown immune responses, although a significant clinical benefit is yet to be shown by large clinical trials.[52] There have been few clinical trials on CEA for pancreatic cancer, but one group[55] has studied a CEA-targeted, autologous dendritic-cell vaccine in three patients with resected pancreatic cancer. Although the sample size was very small, there were no reported toxic effects, and all three patients remain disease-free more than 30 months after surgery.

One clinical trial has assessed the efficacy of vaccination with mutant HRAS peptides in 40 patients with pancreatic cancer of variable stage. Synthetic mutant HRAS polypeptides were processed by presentation through each patient's own antigen-presenting dendritic cells, which were loaded with RAS peptides and then injected intradermally along with granulocyte-macrophage colony-stimulating factor to increase the immune response. Peptide-specific immunity was induced in 58% of patients. Of patients with advanced cancer, those who responded to treatment had longer survival (148 days) than patients who did not respond (61 days, p=0·0002).[56]

Jaffee and colleagues[57] tested a new vaccine in an adjuvant setting in patients with resected pancreatic cancer. Two cell lines of pancreatic cancer were tested, both of which were genetically modified to express the human cytokine granulocye-macrophage colony stimulating factor. The cellular vaccines were administered to 14 patients who had undergone pancreaticoduodenectomy. Delayed-type hypersensitivity in response to the autologous tumour cells occurred in three patients, who also had disease-free survival of longer than 25 months at the time the study was published.[57]

Serial analysis of gene expression and other emerging technology has resulted in the identification of new marker proteins for pancreatic cancer, such as prostate stem-cell antigen,[58] survivin,[59] and mesothelin,[60] that could become targets for immunotherapeutic agents. Mesothelin, a cell-surface glycoprotein expressed in pancreatic cancer, ovarian cancer, and mesotheliomas, is expressed in 89% of pancreatic adenocarcinomas, but not in adjacent healthy pancreatic tissue. A recombinant immunotoxin, SS1 (dsFv)PE38, has been developed to target mesothelin and is presently in phase I trials.[60]

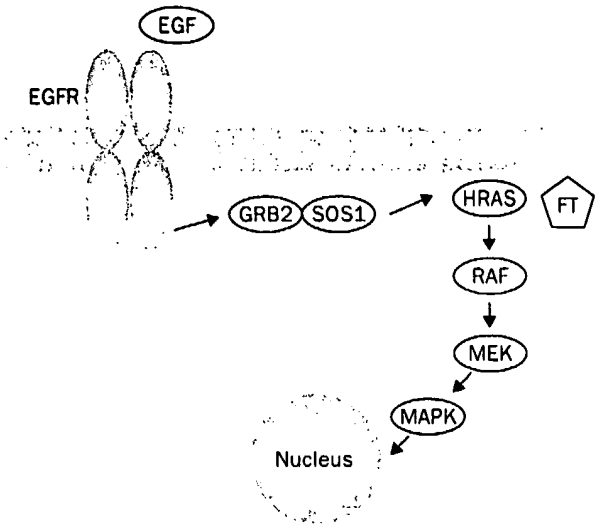

Figure 2. Activation of epidermal-growth-factor receptor (EGFR) stimulates a cascade of downstream kinase proteins. EGF, epidermal-growth factor; FT, farnesyl transferase.

Summary of immunotherapy

Microarray technology and serial analysis of gene expression have identified new targets for vaccine development. However, similar advances are needed in the technical features of vaccine administration and in eliciting the maximum immune response. The most encouraging results in pancreatic cancer have been seen in an adjuvant setting, when there is a lower burden of malignant cells and perhaps less heterogeneity among tumour cells.

Inhibitors of signal transduction

Agents that target EGFR

EGFR, a member of human epidermal receptor family of transmembrane receptor tyrosine kinases, has a crucial role in the growth, repair, and functional differentiation of cells. Receptor tyrosine kinases are overexpressed in many solid tumours, including pancreatic cancer. Activation of the receptors through the binding of ligands (eg, EGF and transforming growth factor α) leads to receptor dimerisation and autophosphorylation, which triggers a signalling pathway that results in proliferation, angiogenesis, and metastasis of tumour cells (figure 2).[61]

Increased expression of EGFR is seen more than 90% of pancreatic-cancer samples[62] and coexpression of EGFR and its ligands in pancreatic cancer is associated with increased tumour size, advanced clinical stage, and poor prognosis.[63]

Monoclonal antibodies directed at EGFR and small-molecule inhibitors of the tyrosine-kinase function of this receptor inhibit the function of the EGFR. The monoclonal antibody cetuximab has been studied the most extensively in clinical trials.

Tumour response and time to progression were assessed in a phase II study of weekly cetuximab with gemcitabine in 41 patients with advanced pancreatic adenocarcinoma who had not previously received chemotherapy, 89% of whom had presence of EGFR confirmed by immunohistochemical analysis. After two courses of treatment five patients achieved a partial response and 16 had stable disease or a minor response. Toxic effects included rash, fatigue, and fever.[64] Median time to progression was 15 weeks and 1-year survival was 32%—results that compare favourably with a previous large phase III trial on a similar group of patients.[2]

Small-molecule inhibitors of EGFR that have been investigated in phase III clinical trials include erlotinib and gefitinib. Both agents are reversible inhibitors of the tyrosine-kinase function of EGFR and have shown activity in vivo in animal studies of human pancreatic cancer. In mice with orthotopic tumours, administration of an EGFR tyrosine-kinase inhibitor decreased tumour size by 45%—an effect that was augmented by the addition of gemcitabine. Therapeutic effects were mediated partly by inhibition of tumour-induced angiogenesis and increased apoptosis.[65]

In a small phase IB clinical trial of erlotinib and gemcitabine in patients with advanced pancreatic cancer or other malignant diseases potentially responsive to this combination, four patients with pancreatic cancer had stable disease and substantial declines in tumour antigen CA19-9, a marker of pancreatic-tumour activity. The most common toxic effects were rash, diarrhoea, fatigue, and neutropenia. Acneiform rash and diarrhoea have consistently been the dose-limiting toxic effects in clinical trials of small-molecule inhibitors of EGFR. A randomised phase III trial in which patients are assigned gemcitabine alone or gemcitabine plus erlotinib is under way.[66]

A phase I multicentre study has assessed the irreversible inhibitor of EGFR, EKB-569, combined with gemcitabine in 29 patients with advanced pancreatic cancer; according to a preliminary report, dose-limiting toxic effects included diarrhoea and increased concentration liver transaminases. One patient has remained on treatment for 8 months, but more data on the efficacy of this combination are needed.[67]

HER2, also a member of the EGFR family, does not have a cognate ligand and instead is a coreceptor that forms heterodimers with other members of the EGFR family, thus stimulating downstream signal-transduction pathways.[68] The humanised monoclonal antibody trastuzumab binds to HER2 and preclinical studies have confirmed its activity in several tumour types that express HER2.[69] Trastuzumab has proven efficacy in the treatment of HER2-positive metastatic breast cancer.

Studies to assess the expression of HER2 in pancreatic cancer have had discrepant results. Gibbs and co-workers[70] analysed a series of 51 resected or biopsy samples of pancreatic tumours for expression of HER2. Suprisingly, only one tumour expressed HER2. Other studies have found HER2 expression in 31 of 151 patients (21%) who were considered for a phase II clinical trial of trastuzumab and gemcitabine. Patients whose tumours overexpressed HER2 were offered the combination regimen. Most recent data from this study shows 22% of patients have had a partial response and 50% a significant reduction in CA19-9.[71]

Intracellular pathways downstream of EGFR

After EGFR binds its ligand, dimerises, and auto-phosphorylates, a cascade of downstream factors is activated. These signals are propagated to the nucleus to affect cellular proliferation, angiogenesis, apoptosis, and metastasis. A well-documented pathway is the GRB2/SOS1/HRAS/RAF1/MAP2K/MAPK pathway and agents that target these signalling proteins are in various stages of clinical development.

A highly selective small-molecule inhibitor of MAP2K called CI-1040 has shown activity in human-cancer xenografts (including pancreatic cancer) and in a phase II clinical trial on 67 patients—15 patients with pancreatic cancer who had not previously received chemotherapy and 52 patients with other tumours (including lung cancer and breast cancer) who had previously been given at least one line of chemotherapy. One patient with pancreatic cancer had stable disease; however, no objective responses were observed.[72]

Further upstream, the HRAS protein controls gene transcription and cellular proliferation. To function in the signal-transduction cascade, HRAS must physically associate with the inner surface of the cell membrane by preylation—the addition of a lipid moiety by the enzyme farnesyl transferase. Thus, farnesyl transferase has become a focus of drug development.[73]

In a phase II trial of initial therapy with the farnesyl-transferase inhibitor tipifarnib in 20 patients with metastatic pancreatic cancer, analysis of peripheral-blood monocytes

Table 4. Clinical trials of targeted agents

Agent	Mechanism	Developmental phase
Flavopiridol (with radiotherapy)	Cyclin-dependent kinase inhibitor	I
SS1 (dsFv)-PE38	Immunotherapy directed at mesothelin protein	I
UCN-01 (with gemcitabine)	Inhibition of protein kinase C	I
Erlotinib (with gemcitabine)	Inhibition of epidermal-growth-factor receptor	I
Bortezomib (with or without gemcitabine)	Proteosome inhibition	II
Triapine (with gemcitabine)	Inhibition of ribonucleotide reductase	II
TNFerade (with fluorouracil and radiotherapy)	Adenovirus-based stimulation of tumour-necrosis-factor secretion	II

Search strategy and selection criteria

Data for this review were identified by searches of PubMed, and the websites of the American Society of Clinical Oncology (from 1995 to April, 2004) and American Association of Cancer Researchers (from 2002 to April, 2004) using the search terms "pancreatic cancer", "gene therapy", "immunotherapy", "angiogenesis", and "signal transduction". References from relevant articles identified by the above search strategy were also used. Only papers published in English, up to April, 2004, were included.

by pharmacodynamic testing showed a decrease in activity of farnesyl transferase. However, the trial could not detect any significant clinical activity for tipifarnib.[74]

A multicentre phase III trial randomly assigned 688 patients with advanced pancreatic cancer to gemcitabine and tipifarnib or gemcitabine and placebo. Median survival did not differ significantly between the two treatment groups; however, patients assigned gemcitabine and tipifarnib had an improved quality of life than did those assigned placebo.[75]

The protein kinase SRC also plays an important part in signal transduction, as well as in the mediation of interactions between pancreatic-cancer cells and the extracellular matrix. A specific inhibitor of SRC has been tested in three human pancreatic-cancer cell lines, in which there was complete inhibition of SRC activity as shown by pharmacodynamic analysis. Moreover, cellular invasiveness was greatly decreased.[76] Other investigations have also confirmed the efficacy of SRC inhibitors in nude mice with pancreatic cancer.[77]

Summary of signal-transduction inhibitors

Although preclinical evidence for the efficacy of signal-transduction inhibitors in pancreatic carcinoma is encouraging, results of clinical trials on single agents have been disappointing. Clinical trials that combine these inhibitors with chemotherapy are under way: like the combination of trastuzumab and paclitaxel in therapy for breast cancer, signal-transduction inhibitors might have greater efficacy if used with standard chemotherapeutic agents.

Conclusion

Most patients with pancreatic cancer have a poor prognosis. Despite advances in the molecular understanding of this malignant disease, initial results from clinical trials of targeted agents have been disappointing. However, encouraging results have been seen in the past year with the development of newer agents such as bevacizumab, the monoclonal antibody to VEGF. Technology continues to advance to methods of delivering gene therapy, antiangiogenic strategies, and identification of immune targets. Clinical trials that combine chemotherapy and signal-transduction inhibitors have shown some benefit in treatment of non-pancreatic cancers, and the results of other combination trials of pancreatic cancer (table 4) are eagerly anticipated.

Conflict of interest

I declare no conflict of interest.

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Clinical picture

Digital gangrene and Raynaud's phenomenon as complications of lung adenocarcinoma

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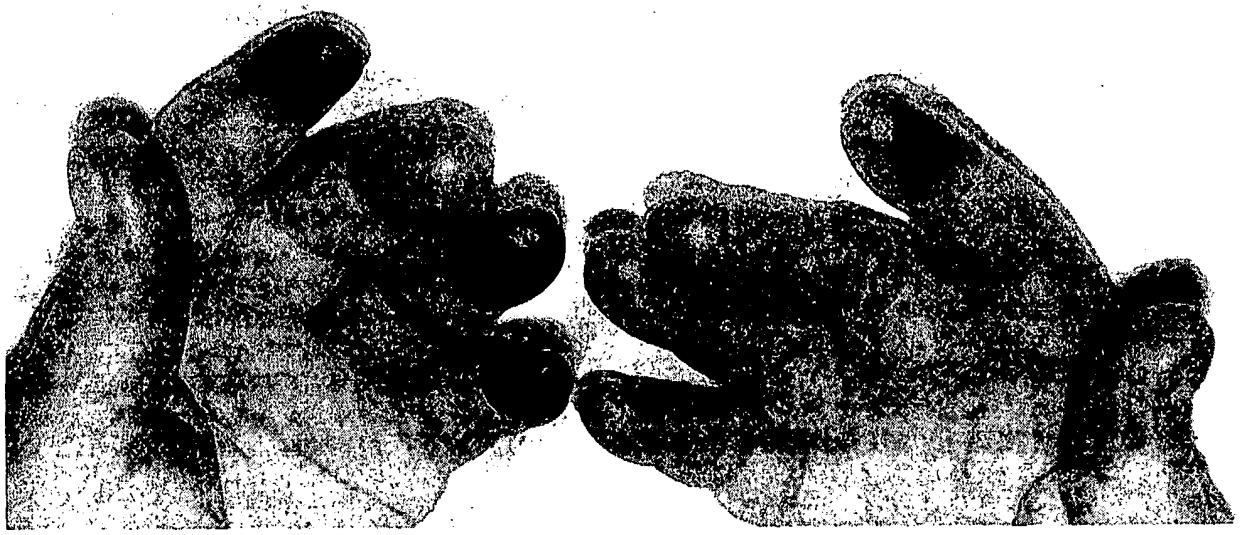

A 65-year-old woman with stage IIIB lung adenocarcinoma that had been diagnosed 6 months earlier presented to our department complaining of severe pain and new-onset discoloration of her fingertips on exposure to cold. A physical examination showed symmetrically normal distal pulses, but most of the fingers were cold and cyanotic from the distal interphalangeal joints to the tips, and four of the fingers were gangrenous (see figure). Extensive work-up including testing for hepatitis viruses B and C, HIV, disseminated intravascular coagulation, antinuclear antibodies, rheumatoid factor, antineutrophil cytoplasmic antibodies, extractable nuclear antigens, anticardiolipin and antiphospholipid antibodies, serum complement concentrations, cold agglutinins, and cryoglobulins remained negative or normal. Echocardiographic analysis ruled out endocarditis. She was diagnosed with paraneoplastic Raynaud's syndrome and given a calcium-channel blocker (nifedipine), unfractionated heparin, aspirin, and intravenous prostacyclin. Despite a very brief improvement in symptoms, the gangrene progressed, and three fingers needed to be amputated. 1 week later, she developed respiratory failure and died.

Abrupt onset of Raynaud's phenomenon and digital ischaemia in elderly patients without abnormal laboratory findings and negative past medical history of thromboembolic disease, arteriosclerotic occlusion, or rheumatological disorders should lead to a thorough search for an underlying malignant disorder.

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Suicide gene therapy with HSV-TK in pancreatic cancer has no effect *in vivo* in a mouse model

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Aim: To study *in vivo* whether pancreatic cancer tumour growth and metastasis can be modified by a gene construct with HSV-TK suicide gene and IL2 co-expression.
Methods: Seventy-eight female SCID mice were i.p. inoculated with retrovirally transduced or control MIA PaCa 2, CAPAN-1 and PANC-1 cell lines. The animals were then randomly selected for saline or ganciclovir (GCV) treatment from the second week, for a total of two weeks.
Results: Most inoculated mice developed tumour nodules and spleen metastases. The liver was colonized by control CAPAN-1 and MIA PaCa 2, but not by PANC-1. Tumours in transduced MIA PaCa 2 cell injected mice were smaller, and in transduced CAPAN-1 injected mice larger, than in control-inoculated mice. There were increased pancreatic and decreased spleen metastases from transduced CAPAN-1, and diminished liver involvement from transduced MIA PaCa 2. No differences were found between mice inoculated with transduced and control PANC-1 cell lines. GCV treatment had no effect on tumour's size or metastases.
Conclusions: The HSV-TK suicide gene does not confer GCV sensitivity to pancreatic cancer in this *in vivo* model. Different pancreatic cancer cell lines cause different growth and metastasis patterns after inoculation in SCID mice, possibly because of variations in their inherent characteristics. The different effects of our vector on cell growth and metastasis may be attributable to the effects of the immunostimulatory cytokine IL2.

Key words: suicide gene therapy; pancreatic cancer; HSV-TK; animal model.

INTRODUCTION

Most pancreatic adeno-carcinomas are refractory to conventional treatment.[1,2] Cancer gene therapy involves suicide genes. A gene encoding for an enzyme which converts a pro-drug into a cytotoxic agent is transferred into the cancer cell.[3–7] The most commonly used suicide gene approach uses the herpes simplex virus-thymidine kinase (HSV-TK). This converts the pro-drug ganciclovir (GCV) into its toxic phosphate derivate. The phosphorylated compound, incorporated into newly synthesized DNA, induces chain termination, and selectively kills dividing cells.[8–12] The HSV-TK system is efficient, and encouraging results have been obtained in several tumours, including pancreatic cancer.[13–18] However, complete tumour eradication is not always achieved because the current vector cannot deliver the functional gene to a sufficient number of cells. The bystander effect, which allows an amplification of the suicide effect, is limited for some tumour cell types.[19–26]

Tumour cells can be genetically engineered to produce various cytokines which enhance the host immune antitumour response. Pancreatic cancer cell lines transduced with interleukin (IL)2, 4, 6, 12, 15, the tumour necrosis factor alpha, or the granulocyte-macrophage colony-stimulating factor, shows an inhibited growth. Some authors have found that they lead to complete tumour regression in subcutaneous nude mice models.[27–32] IL2 and IL4 also provide protection against subsequent re-inoculation of parental pancreatic cell lines.[27] IL2, in particular, is a T-cell growth factor that

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enhances: (1) non-specific immune responses, such as activation of macrophages, natural killer cells, and lymphokine-activated killer cells; (2) major histocompatibility complex-restricted cytotoxic T-cell responses.

The aim of the present study was, therefore, to evaluate whether a gene construct co-expressing human IL-2 and HSV-TK can counteract pancreatic cancer growth and spread in an *in vivo* experimental model.

MATERIALS AND METHODS

Reagents

Dulbecco's modified Eagle's medium (DMEM), RPMI 1640 medium, fetal calf serum (FCS), trypsin, genetycin (G418), superscript II™ reverse transcriptase and gentamycin were purchased from Invitrogen (Italy). Taq DNA polymerase was purchased from Promega (Italy). GCV, high pure RNA isolation kit and XTT assay were purchased from Roche Molecular Biochemicals (Italy). The EIA assay for IL2 measurement was supplied by BioSource Europe S.A. (Italy).

Cell cultures

Three human pancreatic cancer cell lines were used. MIA PaCa 2 and CAPAN-1 were supplied by the American Type Culture Collection (ATCC) and PANC-1, was a gift from Professor Aldo Scarpa, University of Verona-Italy. MIA PaCa 2 and PANC-1 cells were from human primary pancreatic adenocarcinomas, CAPAN-1 cells were from a liver metastasis from pancreatic ductal adenocarcinoma. The cells were kept in continuous culture (75 cm^2 flasks) at 37 °C in a humid atmosphere with 5% CO_2 and 95% air. MIA PaCa 2 cells were grown in DMEM supplemented with 0.1% gentamycin, 2% glutamine and 10% FCS; PANC-1 cells were grown in RPMI supplemented with 0.1% gentamycin and 10% FCS, whereas, CAPAN-1 cells were grown in RPMI supplemented with 0.1% gentamycin and 20% FCS. Cell growth was assessed using the XTT assay. Briefly, for each cell line, 1000 cells were seeded per well in a 96 well culture plate on day 0. XTT assay was made by adding 50 μL reagent to each well, followed by 4 h incubation at 37 °C and by final spectrophotometric reading at 450 nm.

Retroviral vector

The retroviral vectors pLIL-2TKSN and LXSN, donated by Professor Giorgio Palù, Institute of Microbiology, University of Padova-Italy, were Moloney-based retroviral vectors expressing human IL2 and HSV-TK (pLIL-2TKSN) or the only IL2 (LXSN), through a cap-dependent translation and an internal ribosome entry site (IRES)-regulated translation.[33]

Transduction of pancreatic cancer cell lines with the retroviral vector

All pancreatic cancer cell lines were transduced with the vector pLIL-2TKSN. The pancreatic cancer cell line Mia PaCa 2 was also transduced with the vector LXSN. Tumour cells (5×10^5) were plated on 75 cm^2 tissue culture flasks and cultured for 7 days. The culture medium was replaced with 5 mL of retroviral vectors supernatants and polybrene (8 μg/mL) for 4 h. Forty-eight hours after transduction, the cells were selected with a neomycin analogue, genetycin (G418, 0.5–1.5 mg/mL) for 15 days. The surviving colonies were subcultured and established as permanent transduced cell lines.

Detection of HSV-TK gene expression by RT-PCR analysis and IL2 measurement

Total RNA was isolated from pLIL-2TKSN transduced and control cell lines using affinity columns. Reverse transcription was performed using at least 3 μg of total cellular RNA, random hexamer primers (250 ng) and the enzyme, Superscript II™ reverse transcriptase (200 U). Two microlitre of cDNA were added to 23 μL of mixture containing 2.5 μL of 10 × PCR buffer, 1.5 μL of magnesium chloride (25 mM), 1 μL of 5 mM dNTP solution, 1.25 μL each of the forward (5′CTGCGGGTTTATATAGACGG3′) and reverse (5′CATTGTTATCTGGGCGCT3′) TK primers (12.5 pmol), 0.25 μL of Taq DNA polymerase (1.25 U) and 16 μL of H_2O. The PCR cycles were: 94 °C for 3 min; 94 °C for 30 sec; 53 °C for 1 min; and 72 °C for 1 min for 40 cycles; a final extension step was run at 72 °C for 7 min. The PCR product (a 237 bp region of the TK gene) was visualized by electrophoresis on 3% agarose gel stained with ethidium bromide. IL2 protein levels were measured in transduced and non-transduced MIA PaCa 2 cell culture supernatants.

Mouse experiments

The three pLIL-2TKSN and the three control cell lines were tested in mice. Seventy-eight female SCID mice (mean weight 60 g, age six weeks, Charles River Laboratories S.p.A., Milano, Italy) were used for the experiments. All animals received i.p. 5×10^6 transduced or control cells suspended in 500 μL phosphate buffered saline (PBS) at the beginning of the experiments. For each cell line, six groups were identified (Table 1). Starting on day eight of cell inoculation, 500 μL PBS or GCV (100 mg/kg/day) were i.p. injected daily until day 21. The mice were then followed up until day 31 (end of the experiment). At autopsy, any peritoneal tumours were macroscopically and microscopically evaluated and the larger (*A*) and smaller (*B*) diameters measured and

Table 1 Identification of SCID mice experimental groups

Cell lines and number of mice per group (in brackets)	Day of sacrifice	pLIL-2TKSN vector	Treatment
CAPAN-1 ($n = 3$) MIA PaCa2 ($n = 3$) PANC-1 ($n = 3$)	7	Yes	No
CAPAN-1 ($n = 5$) MIA PaCa2 ($n = 5$) PANC-1 ($n = 4$)	30	Yes	PBS
CAPAN-1 ($n = 4$) MIA PaCa2 ($n = 5$) PANC-1 ($n = 4$)	30	Yes	GCV
CAPAN-1 ($n = 3$) MIA PaCa2 ($n = 3$) PANC-1 ($n = 4$)	7	No	No
CAPAN-1 ($n = 3$) MIA PaCa2 ($n = 6$) PANC-1 ($n = 4$)	30	No	PBS
CAPAN-1 ($n = 3$) MIA PaCa2 ($n = 3$) PANC-1 ($n = 5$)	30	No	GCV

For each cell line (CAPAN-1, MIA PaCa 2, and PANC-1), the groups were identified on the basis of pLIL-2TKSN transduced or control injected cells, treatment type and sacrifice time. At the beginning of the experiments all mice received i.p. 5×10^6 pLIL-2TKSN transduced or control cells. PBS or GCV treatments were started on day eight.

recorded. Tumour volume (V; a rotational ellipsoid) was calculated according to the formula: $V(mm^3) = A(mm) \times B^2(mm)^2/2$, in agreement with previous literature reports.[30,34–37] To reduce data dispersion and simplify the analysis, tumour masses were grouped into three main categories: (1) absent; (2) volume less than 60 mm^3; (3) volume greater than 60 mm^3. None of the animals had macroscopically evident metastases. The presence or absence of liver, pancreas, lung, kidney and spleen metastases was verified by microscopy. Eight mice died during the experimental protocol.

Statistical analysis

The statistical analysis of data was made using the analysis of variance, the chi-square test and Fisher's exact test.

RESULTS

Growth and IL2 secretion of transduced and non-transduced cell lines

Figure 1 panel A shows the growth pattern of control MIA PaCa 2 and of the same cell line after transduction with the retroviral vectors, pLIL-2TKSN and LXSN. Although, the two permanently transduced cells lines tended to have a more accelerated growth pattern than control cells, the difference was not statistically significant.

No detectable levels of IL2 protein were found in the control MIA PaCa 2 culture medium, whereas production paralleling cell growth and cell number was found in the culture media of permanently transduced MIA PaCa 2, irrespective of the vector used. Figure 1 panel B shows the pattern of IL2 levels in pLIL-2TKSN MIA PaCa 2.

Figure 1 panel C shows the growth pattern of pLIL-2TKSN transduced and non-transduced CAPAN-1 and PANC-1 cell lines.

Detection of HSV-TK gene expression in cell lines

The expression of HSV-TK mRNA of permanently transduced MIA PaCa 2, CAPAN-1 and PANC-1, before they were inoculated in mice, was evidenced by RT-PCR. All three transduced cell lines expressed HSV-TK mRNA, which appeared as a 237 bp product after agarose gel elecrophoresis.

Results on day 7

Table 2 shows the results obtained after one week from pLIL-2TKSN transduced or control cell lines inoculation. The number of mice developing or not developing tumour masses, liver or spleen metastases are reported together with a statistical analysis of data comparing, for each cell line, pLIL-2TKSN transduced and control injected mice. Sixty-six percent of MIA PaCa 2 injected mice did not develop primary tumour masses. All CAPAN-1 and 86% of PANC-1 injected mice developed

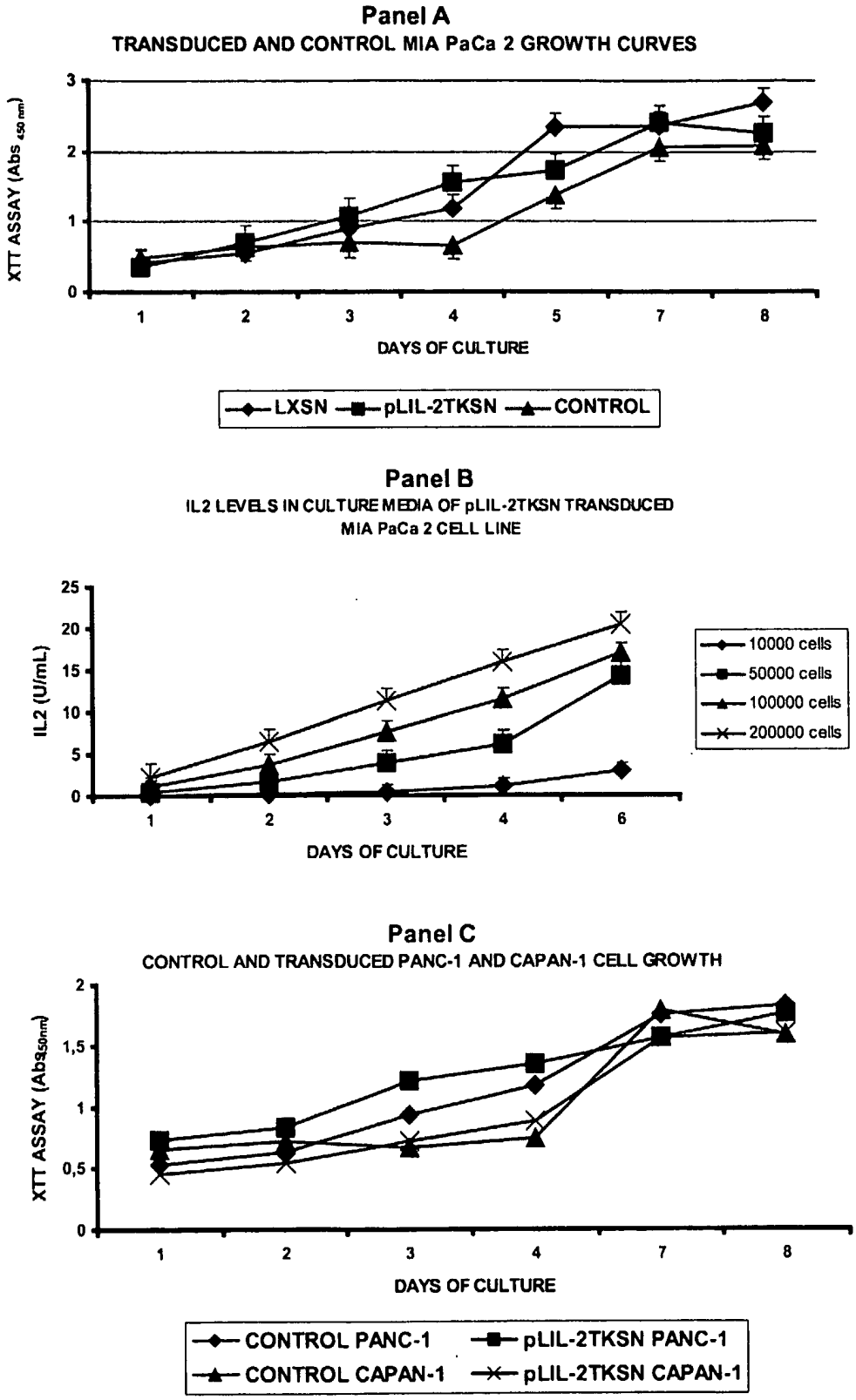

Figure 1 Panel A: Growth curves of non-transduced (control), LXSN and pLIL-TKSN-transduced MIA PaCa 2 cell lines. Bars represent standard errors. Panel B: IL2 levels measured in tissue culture media of pLIL-2TKSN transduced MIA PaCa 2 cell lines. A different number of cells were seeded in a 24 well culture plate. Bars represent standard errors. Panel C: Growth curves of non-transduced (control) and pLIL-2TKSN transduced PANC-1 and CAPAN-1 cell lines.

Table 2 Findings on day seven

Cell line (overall no. mice)	Tumour masses			Hepatic metastases		Splenic metastases	
	Absent	<60 mm^3	>60 mm^3	Present	Absent	Present	Absent
Control CAPAN-1 ($n = 3$)	0	2	1	2	1	0	3
pLIL-2TKSN CAPAN-1 ($n = 3$)	0	2	1	0	3	1	2
Fisher's exact test		p : NS		p : NS		p : NS	
Control PANC-1 ($n = 4$)	0	2	2	0	4	2	2
pLIL-2TKSN PANC-1 ($n = 3$)	1	1	1	0	3	1	2
Fisher's exact test		p : NS		p : NS		p : NS	
Control MIA PaCa 2 ($n = 3$)	2	1	0	0	3	3	0
pLIL-2TKSN MIA PaCa 2 ($n = 3$)	2	1	0	0	3	3	0
Fisher's exact test		p : NS		p : NS		p : NS	

At the beginning of the experiment all mice received i.p. 5×10^6 pLIL-2TKSN transduced or control cells. No treatment was performed during the first week. The absolute number of mice that did not develop, or developed tumour masses with a volume less or larger than 60 mm^3, liver or spleen metastases are reported. The statistical analysis of data was made comparing, for each cell line, pLIL-2TKSN transduced and control injected mice.

peritoneal tumours one week after inoculation. In none of the cell lines studied was a difference found between transduced and control injected mice considering for tumour masses. Unlike most transduced or control CAPAN-1 injected mice, transduced or control MIA PaCa 2 mice developed spleen metastases. Liver metastases were found in 66% of control CAPAN-1 injected mice.

No mice had pancreatic, lung and kidney metastases after one week.

Results on day 30

None of the mice had lung or kidney metastases.

Results for mice injected with control cell lines after PBS treatment: the CAPAN-1 and MIA PaCa 2 cell lines caused more frequent liver metastases (66.6%) than PANC-1 (0%). In MIA PaCa 2 injected mice, spleen metastases were more frequent (83.3%) than in the other two lines (66.6% for CAPAN-1 and 50% for PANC-1). All MIA PaCa 2 injected mice had tumours larger than 60 mm^3, while they were found in 50% of PANC-1 and 33% of CAPAN-1 injected mice. Pancreatic metastases were recorded in 100% of CAPAN-1, 50% of PANC-1 and 83% of MIA PaCa 2 injected mice.

Figure 2 shows the results for tumour masses in transduced and control cell lines injected mice after PBS treatment. CAPAN-1, MIA PaCa 2 and PANC-1 results are reported in panels A, B and C, respectively. $\chi^2 = 4.44$, $p < 0.05$ for CAPAN-1, $\chi^2 = 11.0$, $p < 0.01$ for MIA PaCa 2, $\chi^2 = 2.33$, p : NS for PANC-1. In transduced CAPAN-1 injected mice, all tumours were >60 mm^3 (100%) in volume, unlike in control CAPAN-1 injected mice ($\chi^2 = 8.0$, $p < 0.05$) (panel A). In mice injected with the transduced or control PANC-1 cell line, no differences were found for tumour masses ($\chi^2 = 2.33$, p : NS) (panel B). In transduced MIA PaCa 2 injected mice, tumours were absent (60%) or less than 60 mm^3 (40%) in volume, unlike in control MIA PaCa 2 mice, all of which presented tumours larger than 60 mm^3 ($\chi^2 = 11.0$, $p < 0.01$) (panel C).

Figure 3 shows the results for liver, spleen and pancreatic metastases in transduced and control cell lines injected mice after PBS treatment. CAPAN-1, MIA PaCa 2 and PANC-1 results are reported in panels A, B and C, respectively. The percentages of mice developing liver (L), spleen (S) or pancreatic (P) metastases are reported. In mice injected with transduced or control CAPAN-1, a significant difference was found in the development of spleen ($\chi^2 = 4.44$, $p < 0.05$) and pancreatic ($\chi^2 = 4.8$, $p < 0.05$), but not of liver metastases ($\chi^2 = 1.9$, p : NS) (panel A). No significant difference was found for any parameter considering PANC-1 cells (panel B). In transduced MIA PaCa 2 injected mice, no liver metastases were found, unlike in control MIA PaCa 2 injected mice, 36.3% of which presented liver metastases ($\chi^2 = 5.24$, $p < 0.05$). No significant differences were found when spleen and pancreatic metastases were considered (panel C).

GCV treatment had no effect on tumour volume, liver and spleen metastases of mice injected with pLIL-2TKSN transduced CAPAN-1, PANC-1 or MIA PaCa 2 cells.

DISCUSSION

The features of pancreatic cancer include cell cycle regulator alterations, with mutations of oncogens and tumour suppressor genes and the ability to escape the immune surveillance, allowing tumour cells to grow and metastasize[38,39] The novel gene therapy approach investigated in the present paper allows the insertion of DNA sequences into the tumour cell genome, with a view to counteracting tumour growth and spread, conferring tumour cell sensitivity on chemotherapeutical

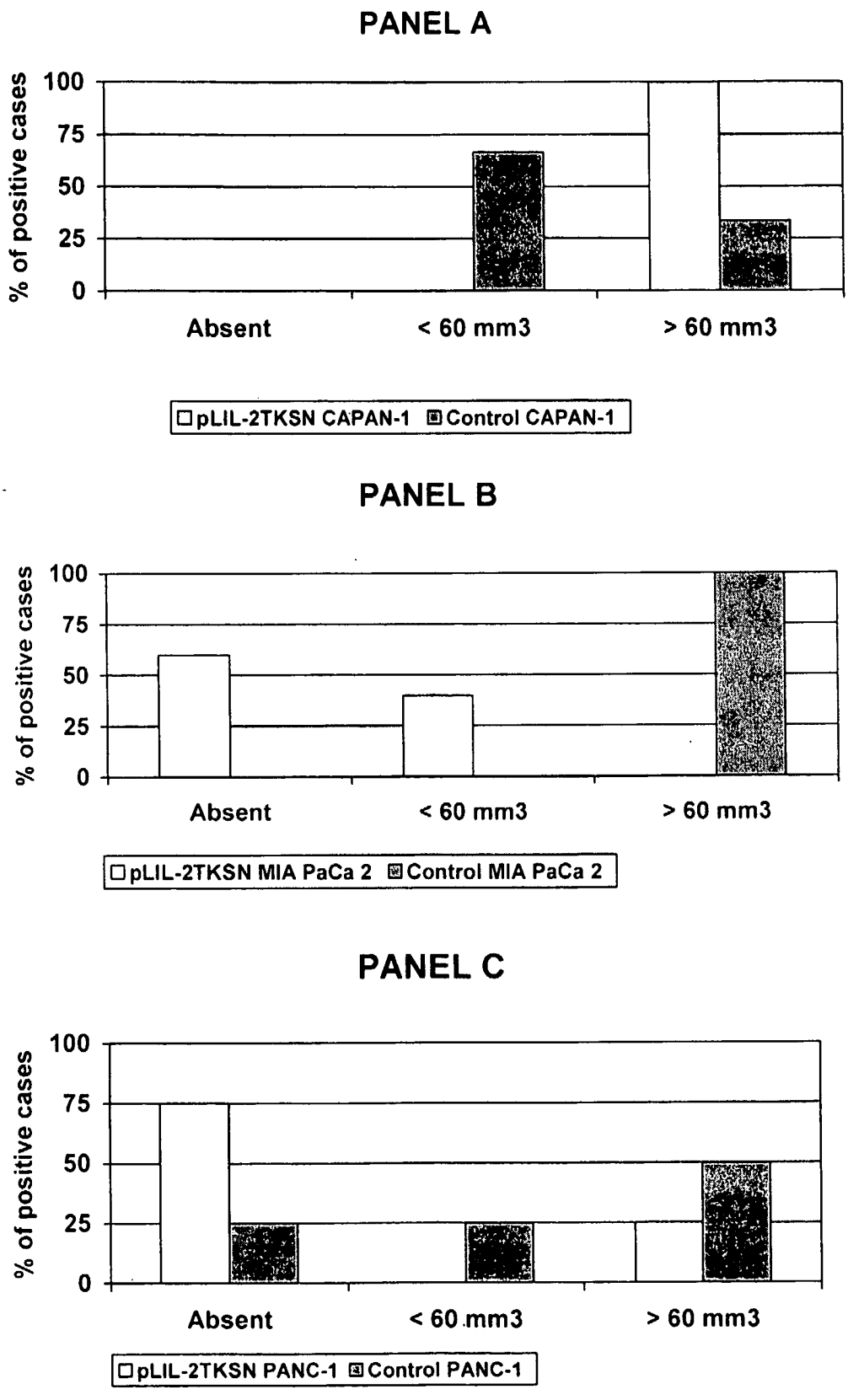

Figure 2 Results at one month for tumour masses in pLIL-2TKSN transduced and control injected mice after PBS treatment.

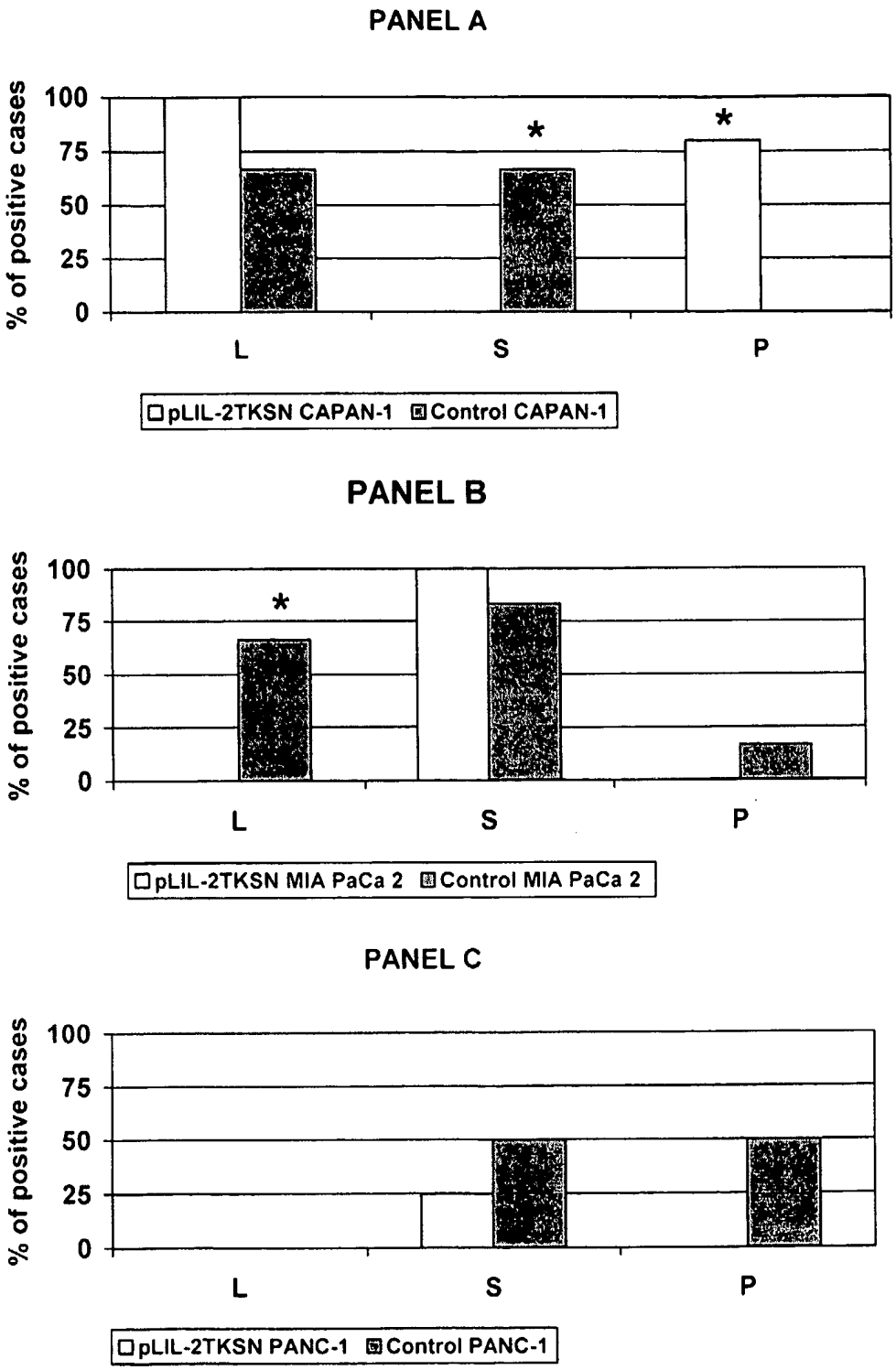

Figure 3 Results at one month for hepatic (L), splenic (S) and pancreatic (P) metastases in pLIL-2TKSN-transduced and control injected mice after PBS treatment. Fisher's exact test: * = $p < 0.05$.

agents and/or enhancing the host immune response. In theory, a gene construct consisting of more than one gene, and acting on different targets, should have an enhanced therapueutic potential. We verified *in vivo* the effects of a gene construct consisting of the suicide gene HSV-TK and the immunostimulatory cytokine, IL2, which was retrovirally transduced into three pancreatic cancer derived cell lines with different metastatic potentials in order to cover, at least in part, the broad spectrum of biological variability of this tumour type. A vector expressing only IL2 was also tested *in vitro* in one of these lines, MIA PaCa 2. The *in vitro* growth patterns of

MIA PaCa 2 non-transduced and transduced with the two vectors were quite similar, although the two transduced lines exerted growth that was slightly faster than that in control line (Fig. 1). Furthermore, the two transduced lines released similar amounts of IL2 in culture media. This suggests that the presence of the HSV-TK gene in the vector does not interfere with IL2 transcription and translation.

HSV-TK, a suicide gene, causes transfected cells to be highly sensitive to the pro-drug, GCV.[5,8,28] In several tumour types, including pancreatic cancer, the HSV-TK gene inhibits cellular growth after GCV treatment *in vitro*,[12,17,25,40] although this effect cannot be reproduced in all pancreatic cancer derived cell lines.[11,19–21,40] A controversial issue is the transferral of *in vitro* data into *in vivo* applications.[34,41] SCID mice were used in order to obviate any immunological response to foreign cells. Before inoculation in mice, used RT-PCR to ascertain whether the new transduced TK gene was correctly translated into the corresponding mRNA. The results obtained indicate that all transduced lines expressed the suicide gene and should, therefore, be sensitive to GCV.

All mice were i.p. inoculated with pLIL-2TKSN transduced and control cell lines. Nineteen mice were sacrificed after one week, before any treatment, to evaluate: (1) the starting tumour mass and its spread; and (2) any differences between transduced and control injected mice. No differences were observed, and this indicates that early *in vivo* growth and spread are not influenced by HSV-TK or by IL2, co-expressed by our vector. On considering the different pancreatic cancer cell lines, differences in tumour growth pattern clearly emerged just after one week: in all mice, the metastasis derived CAPAN-1 line produced evident tumour nodules, while the primary line MIA PaCa 2 caused tumour onset in only a few mice (2/6). An intermediate pattern was observed when PANC-1 cells were inoculated. The spleen was rapidly colonized by MIA PaCa 2 (all mice) and PANC-1 (3/7 mice), but not by CAPAN-1 (1/6), which, however, rapidly colonized the liver (2/6 mice). In all injected mice, the pancreas was metastases free. The above findings may have depended on different cell lines causing different tumour types due to their inherent characteristics: the high hepatic tropism in CAPAN-1 cells may have depended on their human preselection, since they were isolated from liver metastases from pancreatic cancer. The high splenic tropism of MIA PaCa 2, and the high hepatic tropism of CAPAN-1 cells were confirmed at the end of experiments considering control cells. Furthermore, the liver was targeted by MIA PaCa 2 cells while in no cases did PANC-1 metastasize to the liver. The similarity between the CAPAN-1 and MIA PaCa 2 metastatic patterns was further observed on considering pancreatic involvement at the end of the experimental protocol, which was recorded in none of the CAPAN-1 injected mice, and in only 1/6 MIA PaCa 2 injected mice, while it was the target in 50% of PANC-1 injected mice.

Interestingly, our pLIL-2TKSN vector modified 'per se' the behavior of MIA PaCa 2 and CAPAN-1 transduced pancreatic cancer cell lines 30 days after i.p. inoculation: on comparing tumour growth and spread in mice inoculated with transduced pancreatic cancer cell lines and saline treated with patterns in mice inoculated with control cell lines, we found: (1) smaller tumour volumes in mice injected with transduced MIA PaCa 2 cells, unlike in transduced CAPAN-1 injected mice; (2) increased pancreatic and decreased spleen metastases from transduced CAPAN-1, and diminished liver involvement from transduced MIA PaCa 2. No differences were found between mice inoculated with transduced and those inoculated with control PANC-1 cell lines. Since these effects were correlated with the vector itself, rather than with the sensitivity conferred by it on GCV, the above effects probably depended on IL2. This cytokine is known to stimulate the specific immune response in immunocompetent animals, but it also causes non-specific activation of the macrophages as well as the natural killer cells, both of which are borne by SCID mice.[29–32] Our results can be explained only in part by IL2 activation of these cells. If IL2 activation of macrophages and natural killer cells had an anti-neoplastic effect in SCID mice, all pLIL-2TKSN injected mice are expected to have smaller tumours and fewer metastases than control injected mice. As this was not the case, we believe that IL2 probably acts directly in an autocrine manner on tumour cells, possibly evoking a different response depending on the tumour cell status, as suggested by other authors on analysing the effects of TGF-β1.[42,43] This response pattern, however, does not seem to comprise cell growth, which was unaffected *in vitro* (Fig. 3).

HSV-TK did not lead to cellular death after GCV treatment. None of the analysed parameters (tumour volume, liver, spleen and pancreatic metastases) were modified after GCV treatment in pLIL-2TKSN cell injected mice. The HSV-TK gene cannot, therefore, be considered a suicide gene for pancreatic cancer, probably because pancreatic cancer cells can escape the lethal consequences of GCV, possibly by rapidly metabolizing this compound. This pro-drug is, in fact, first phosphorylated by the HSV-TK enzyme. The resultant GCV-monophosphate is subsequently converted by cellular kinases into the toxic GCV-triphosphate nucleotide, a substrate for DNA polymerase. Any alteration in cellular kinases, often found in tumour cells, might compromise GCV activation, and therefore, disable any HSV-TK suicide effect.[19–21]

In conclusion, *in vivo* suicide gene therapy with HSV-TK for pancreatic cancer has failed to meet expectations. A search should, therefore, be made for alternative therapeutic genes in the attempt to cure this disease.

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Killer genes in pancreatic cancer therapy.

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This review describes: 1. The main genetic alterations found in pancreatic cancer (EGF-R overexpression, SST-2 somatostatin receptor loss of expression, k-ras, p53 mutations and DPC4 mutations) and the effect of their replacements by gene therapy on tumor growth; 2. The use of suicide genes (HSV-TK and CD) for pancreatic cancer gene therapy in vitro and in vivo; 3. The implications for pancreatic cancer treatment when using cytotoxic bacterial toxins; 4. Viral and non-viral delivery systems for the transfer of therapeutical genes into pancreatic cancer cells. Overall both the correction of pancreatic cancer cells main genetic alterations and the use of suicide genes allow only partial tumor regression in vitro and in vivo. The lack of a 100% effect for any studied strategy considered alone, indicates the need for combined therapies to achieve a satisfactory treatment of this tumor.

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Suicide gene therapy for urogenital cancer: current outcome and prospects.

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Viral-mediated transfer of the herpes simplex virus thymidine kinase (HSV-tk) gene has been demonstrated by several investigators to confer sensitivity to nucleoside analogs such as ganciclovir (GCV) in a variety of tumor cells including brain, prostate, bladder, kidney, ovary, head and neck, lung, pancreas, and liver cancers. Fourteen suicide gene clinical protocols using adenovirus vectors have been conducted, including four in prostate cancer. Two additional protocols for prostate cancer are in preparation in Japan and the Netherlands. A study conducted at Baylor College of Medicine was the first to demonstrate the safety of HSV-tk plus GCV therapy for human prostate cancer and the anticancer activity of gene therapy in this disease. However, it is still in the early stage of its development, with a number of problems to be overcome. Systemic delivery, specific introduction, and specific expression of the target gene are the major issues to be managed in order to establish a clinically relevant treatment strategy.

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